2020-1785

United States Court of Appeals for the Federal Circuit

BIO-RAD LABORATORIES, INC.,
Appellant

v.

INTERNATIONAL TRADE COMMISSION,
Appellee

10X GENOMICS, INC.,
Intervenor

Appeals from the United States International Trade Commission in Investigation No. 337-TA-1100.

NONCONFIDENTIAL PRINCIPAL BRIEF FOR APPELLANT BIO-RAD

Brian C. Cannon
Kevin P.B. Johnson
QUINN EMANUEL URQUHART & SULLIVAN, LLP
555 Twin Dolphin Drive, 5th Floor
Redwood Shores, California 94065
Telephone: (650) 801-5000
briancannon@quinnemanuel.com
kevinjohnson@quinnemanuel.com

Counsel for Appellant Bio-Rad Laboratories, Inc.

David Bilsker
Nathan Sun
Andrew Naravage
QUINN EMANUEL URQUHART & SULLIVAN, LLP
50 California Street, 22nd Floor
San Francisco, California 94111
Telephone: (415) 875-6600
davidbilsker@quinnemanuel.com
nathansun@quinnemanuel.com
andrewnaravage@quinnemanuel.com

S. Alex Lasher
QUINN EMANUEL URQUHART & SULLIVAN, LLP
1300 I Street NW, Suite 900
Washington, DC 20005
Telephone: (202) 538-8104
alexlasher@quinnemanuel.com

Sean T. Gloth
QUINN EMANUEL URQUHART & SULLIVAN, LLP
51 Madison Avenue, 22nd Floor
New York, New York 10010
Telephone: (212) 849-7468
seangloth@quinnemanuel.com

Counsel for Appellant Bio-Rad Laboratories, Inc.

LANGUAGE OF A PATENT CLAIM AT ISSUE

Claim 1 of United States Patent No. 9,689,024 provides:

1. A method for sample preparation, comprising:

a) providing a droplet comprising a porous gel bead and a target nucleic acid analyte, wherein said porous gel bead comprises at least 1,000,000 oligonucleotide molecules comprising barcode sequences, wherein said oligonucleotide molecules are releasably attached to said porous gel bead, wherein said barcode sequences are the same sequence for said oligonucleotide molecules;

b) applying a stimulus to said porous gel bead to release said oligonucleotide molecules from said porous gel bead into said droplet, wherein upon release from said porous gel bead, a given oligonucleotide molecule from said oligonucleotide molecules attaches to said target nucleic acid analyte; and

c) subjecting said given oligonucleotide molecule attached to said target nucleic acid analyte to nucleic acid amplification to yield a barcoded target nucleic acid analyte.

CERTIFICATE OF INTEREST

Counsel for the appellant Brian C. Cannon certifies the following:

1. Full Name of Party Represented by me:

Bio-Rad Laboratories, Inc.

2. The name of the real party in interest (if the party named in the caption is not the real party in interest) represented by me is:

None/Not Applicable.

3. All parent corporations and any publicly held companies that own 10 percent or more of the stock of the party represented by me are:

None/Not Applicable.

4. The names of all law firms and the partners or associates that appeared for the party or amicus now represented by me in the trial court or agency or are expected to appear in this court (and who have not or will not enter an appearance in this case) are:

Quinn Emanuel Urquhart & Sullivan, LLP – Peter Benson, Jeffrey Gerchick, Ethan Glass, Victoria Maroulis, Anne S. Toker, Nancy Zhang

Sky C. Adams, Jeffrey Ung, formerly of Quinn Emanuel Urquhart & Sullivan, LLP

5. The title and number of any case known to counsel to be pending in this or any other court or agency that will directly affect or be directly affected by this court's decision in the pending appeal. *See* Fed. Cir. R. 47. 4(a)(5) and 47.5(b).

10X Genomics, Inc. v. Bio-Rad Laboratories, Inc., Case No. 4:18-cv-00209-JST (N.D. Cal.)

6. Any information required under Fed. R. App. P. 26.1(b) (organizational victims in criminal cases) and 26.1(c) (bankruptcy case debtors and trustees). Fed. Cir. R. 47.4(a)(6).

None/Not Applicable.

Date: August 17, 2020

/s/ Brian C. Cannon
Brian C. Cannon

TABLE OF CONTENTS

Page

This brief contains information that is confidential and that has been removed from this nonconfidential version of the brief pursuant to Federal Circuit Rule 25.1(e). The confidential material omitted from page 42 concerns a confidential chemical structure used by Bio-Rad Laboratories, Inc. in its products.

TABLE OF AUTHORITIES

Page

Cases

Statutory Authorities

Additional Authorities

STATEMENT OF RELATED CASES

Appellant identifies the following appeals as related: None.

PRELIMINARY STATEMENT

This is an appeal from a final determination of the International Trade Commission ("ITC") issuing an exclusion order and cease and desist order against Bio-Rad for infringement of three patents related to droplet-generating microfluidic devices used to prepare samples for single-cell DNA analysis and related applications. The ITC's decision should be reversed because Bio-Rad owns a share of each of the three patents, and 10X failed to prove infringement and domestic industry.

One way to prepare genetic material for next generation sequencing involves isolating individual cells and delivering reagents in order to tag—or "barcode"—DNA from each cell. Microscopic droplets can be used to partition cells and reagents. Each asserted patent is directed to generating such droplets.

Two of the six inventors on the three patents are former Bio-Rad employees. At Bio-Rad and its predecessor QuantaLife, the two named inventors conceived of ideas that are reflected in the asserted patents. For instance, while at QuantaLife, they presented slides internally revealing that "[d]roplets can be tagged for nextgen sequencing to obtain single cell-level resolution." Appx03442. At QuantaLife, they conceived of a reagent delivery system and the idea to use oligonucleotides as barcodes to tag sample DNA. All of these ideas belong to Bio-Rad pursuant to these inventors' assignment agreements.

About a year after selling QuantaLife to Bio-Rad for more than $160 million, the two inventors left Bio-Rad to form 10X. Then, in less than four months, they filed applications leading to the asserted patents. Under well-established ownership and inventorship principles, Bio-Rad owns a pro rata share of the patents based on the work that two of the six inventors conceived at QuantaLife/Bio-Rad. Their work was not a vague hope for a future research plan, but a specific solution to a problem using specific reagents. At a minimum, their work met the threshold required for co-inventorship. The error below was to require a single "eureka" moment contrary to the controlling contractual language; the ITC ignored that multiple people can contribute to patented inventions over time and that co-inventors each do not have to conceive all elements of a patent claim.

Separately, 10X failed to prove infringement of each of the three patents. This is because the claims as written reflect the state of the project when the two inventors left Bio-Rad. After the two inventors left, however, Bio-Rad developed the accused products and followed a path that take them out of the scope of the claims.

For the '024 Patent, the stimulus Bio-Rad uses to release reagents within the droplets is not the claimed stimulus that acts on the internal delivery mechanism (the claimed porous gel bead), but rather, an enzyme that destroys part of the oligonucleotide barcode molecule attached to the bead. For the '468 Patent, Bio-Rad's products do not contain the claimed "aqueous mixture" on the microfluidic

device before the droplets form because in Bio-Rad's system, the reagents act immediately and must be kept separate from the sample until droplets form. And for the '530 patent, there is no evidence supporting a finding that the relevant systems detach barcodes and barcode DNA after at least 1,000 droplets are formed as the construed claims require because the barcode detachment and barcoding occurs immediately as each droplet forms. In addition, for the '530 Patent, the conflicting claim constructions below regarding the order of steps to be followed in the claimed method demonstrate the claims are indefinite.

JURISDICTIONAL STATEMENT

This is an appeal from a final determination of the International Trade Commission under 19 U.S.C. § 1337. The Commission issued its final determination on February 12, 2020. Bio-Rad timely filed its petition for review on May 8, 2020. *See* 19 U.S.C. § 1337(c). This Court has jurisdiction under 28 U.S.C. § 1295(a)(6).

STATEMENT OF THE ISSUES FOR APPEAL

1. Whether the Commission erred in requiring complete conception of all elements of the claimed inventions in the Asserted Patents by all inventors before any contractual rights could attach, and erred in determining that Bio-Rad failed to show that it has a pro rata ownership interest in the Asserted Patents.

2. Whether the Commission erred in finding Bio-Rad infringes the '024

Patent where the claim requires (a) an oligonucleotide "releasably attached" to a porous gel bead and (b) a "stimulus" be applied to the gel bead—but where the alleged stimulus in Bio-Rad's products is applied to the oligonucleotide itself rather than to the gel bead.

3. Whether the Commission erred in finding Bio-Rad infringes the '468 Patent where all evidence demonstrates no "aqueous mixture" exists at a first junction in a microfluidic device prior to droplet generation at a second junction.

4. Whether the Commission erred in finding a violation with respect to the '530 Patent where (a) no evidence shows that barcodes detach from gel beads in at least 1,000 droplets after those droplets are formed in either 10X's domestic industry or Bio-Rad's accused products, and (b) the Commission adopted contradictory claim constructions that demonstrate the claims are indefinite.

STATEMENT OF THE CASE

A. Overview Of The Technology

This case concerns preparing samples for next generation DNA sequencing. The method allows the material in each cell to be studied individually, rather than having to study genetic material from a sample in bulk as was commonly done before the invention. This allows researchers to study genetic differences on a cell-by-cell basis, and identify, for example, certain variations present in one cancer cell from a sample that is not seen in another cell from the same sample.

In one form of preparing DNA for next generation sequencing, the idea is to use partitions to isolate single cells and introduce reagents that will extract the DNA and tag it. The tagging process tracks each individual cell's DNA through subsequent steps such as PCR amplification (to create multiple copies)[1] and sequencing.

A critical component of this preparation method for next generation sequencing is the ability to partition the sample by using microscopic droplets, each of which acts as a "mini-test tube." *See, e.g.*, *Bio-Rad Labs., Inc. v. 10X Genomics Inc.*, No. 2019-2255, 2020 WL 4431893, at *1 (Fed. Cir. Aug. 3, 2020) ("Each droplet holds a single cell and the required reagents for the biochemical reaction."). Microfluidic devices—often called "chips"—include channels, or "hair-width pathways through which cells and fluids flows." *Id*. The chips form droplets by "pinching off" the aqueous sample fluid within an immiscible oil fluid. *Id*.

Another critical component is the use of reagents called "barcodes" to tag the genomic material of the individual cells, and a reagent delivery system to bring the barcodes in contact with the sample DNA within each droplet.

[1] PCR refers to the polymerase chain reaction, a technique to make millions of copies of a particular piece of DNA. *See, e.g.*, *Roche Molecular Sys., Inc. v. CEPHEID*, 905 F.3d 1363, 1366 (Fed. Cir. 2018). This is referred to as amplification of DNA. The process involves heating and cooling, called thermal cycling, on a purpose-built machine. Both Bio-Rad's and 10X's systems use a thermocycler to amplify DNA after the sample tagging process.

B. The Patents-In-Suit

10X asserts U.S. Patents Nos. 9,689,024 (the '024 Patent); 9,695,468 (the '468 Patent); and 9,856,530 (the '530 Patent). Appx00346; Appx00377; Appx00408.

There is commonality across each of the three 10X patents-in-suit. Each is directed to the use of droplets to partition a sample; each requires oligonucleotides as barcodes; and each has a reagent delivery system, namely porous gel beads to which the oligonucleotides are releasably attached.

1. The '024 Patent: Stimulus Applied To Gel Bead

Asserted claim 1 of the '024 Patent is directed to a method for sample preparation involving the steps of providing a droplet, within which is a porous gel bead with releasably attached oligonucleotides to serve as barcodes. The disputed aspect of this claim is that the stimulus to release the barcodes must be applied to the gel bead. Claim 1 of the '024 Patent provides:

> 1. A method for sample preparation, comprising:
>
> a) providing a droplet comprising a porous gel bead and a target nucleic acid analyte, wherein said porous gel bead comprises at least 1,000,000 oligonucleotide molecules comprising barcode sequences, wherein **said oligonucleotide molecules are releasably attached to said porous gel bead**, wherein said barcode sequences are the same sequence for said oligonucleotide molecules;
>
> b) **applying a stimulus *to said porous gel bead* to release said oligonucleotide molecules from said porous gel bead** into said

> droplet, wherein upon release from said porous gel bead, a given oligonucleotide molecule from said oligonucleotide molecules attaches to said target nucleic acid analyte; and
>
> c) subjecting said given oligonucleotide molecule attached to said target nucleic acid analyte to nucleic acid amplification to yield a barcoded target nucleic acid analyte.

Appx00375 at 33:55-34:7 (Claim 1).[2] In Bio-Rad's system, the stimulus is applied to the oligonucleotide, not the gel bead. *Infra* Part II.

2. The '468 Patent: "Aqueous Mixture" Between Two Junctions

The '468 Patent is directed to a method of creating an "aqueous mixture" from two aqueous streams—one that contains barcodes releasably attached to a gel bead, and another that contains the sample and other reagents—and then encapsulating that aqueous mixture in a droplet:

> 1. A method for droplet generation, comprising:
>
> (a) providing at least 1,000,000 oligonucleotide molecules comprising barcode sequences, wherein said barcode sequences are the same sequence for said at least 1,000,000 oligonucleotide molecules, wherein said at least 1,000,000 oligonucleotide molecules are releasably attached to a bead, wherein said bead is porous;
>
> (b) combining said at least 1,000,000 oligonucleotide molecules and a sample comprising a nucleic acid analyte each in an aqueous phase at a **first junction** of two or more channels of a microfluidic device to form an ***aqueous mixture*** comprising said at least 1,000,000 oligonucleotide molecules attached to said bead and said sample; and
>
> (c) generating a droplet comprising said at least 1,000,000

[2] All emphases are added unless otherwise specified.

> oligonucleotide molecules attached to said bead and said sample comprising said nucleic acid analyte by contacting ***said aqueous mixture*** with an immiscible continuous phase at a **second junction** of two or more channels of said microfluidic device.

Appx00406 at 33:55-34:9 (Claim 1). In Bio-Rad's system, the reagent fluid and sample fluid do not mix before droplets form. *Infra* Part III.

3. The '530 Patent: As Construed, Requires Steps Followed In Order

Asserted claim 1 of the '530 Patent is a three-step method for nucleic acid analysis. The appeal issue is the order of steps and the numerical limitation of detaching a plurality of barcodes and barcoding a plurality of polynucleotides in each of at least 1,000 droplets after all 1,000 droplets are created. Claim 1 provides:

> 1. A method for nucleic acid preparation or analysis, comprising:
>
> (a) providing:
>
> (i) at least 1,000 gel beads;
>
> (ii) releasably attached to each of said at least 1,000 gel beads, at least 1,000 barcode molecules comprising identical barcode sequences that are distinct from barcode sequences of at least 1,000 barcode molecules releasably attached to any other gel bead of said at least 1,000 gel beads; and
>
> (iii) a plurality of cells each comprising a plurality of polynucleotide molecules;
>
> (b) ***generating*** a plurality of droplets, ***wherein at least 1,000 droplets of said plurality of droplets each comprise***:
>
> (i) a single gel bead from said at least 1,000 gel beads; and

(ii) a single cell from said plurality of cells; and

(c) ***in each of said at least 1,000 droplets***, using said plurality of polynucleotide molecules from said single cell and barcode molecules of said ***at least 1,000 barcode molecules*** from said single gel bead to generate a plurality of barcoded polynucleotide molecules,

wherein said barcode molecules become detached from said gel bead.

Appx00450-00451 at 47:58-49:4 (Claim 1). The Commission ruled: "The process requires . . . that cleavage and barcoding occur in ***at least 1,000 droplets*** after those droplets are generated." Appx00080. Because the beads in the 10X system, and the oligonucleotides in the Bio-Rad system, each release their barcodes immediately when each droplet is formed, there is no evidence that a plurality of barcodes detach from each bead and barcode a plurality of polynucleotides in each of at least 1,000 droplets ***after*** at least 1,000 droplets are generated. *Infra* Part IV.

C. Bio-Rad's Accused Products

In 2011, Bio-Rad acquired a microfluidics startup company called QuantaLife for approximately $160 million, including all its assets and intellectual property. Appx00260; Appx03178. Shortly after acquiring QuantaLife, Bio-Rad commercialized part of the microfluidics intellectual property. Bio-Rad's first droplet product was called Droplet Digital™ PCR (ddPCR™), which launched in 2011. The DNA fragments encapsulated in the droplets from these devices then undergo analysis by digital PCR on a thermocycler. Appx10036 at 138:1-21.

At the time of Bio-Rad's acquisition, QuantaLife employed Serge Saxonov and Benjamin Hindson. Appx00673. Saxonov and Hindson worked at Bio-Rad for about a year before leaving to found 10X. Within four months of leaving Bio-Rad, Saxonov and Hindson named themselves as inventors on a patent application that would underlie the patents now being asserted against Bio-Rad. Appx00673-00674; Appx00299; Appx00346, Appx00377, Appx00408.

After Saxonov and Hindson left, Bio-Rad's development work continued. Appx03180-03181. Bio-Rad eventually released its single cell ddSEQ system in 2017. Appx10061 at 238:23-25. In the underlying Investigation before the Commission, 10X accused Bio-Rad's ddSEQ system, including various chips, instruments, and reagents, of infringing the 10X patents. Appx00543-00544 ¶ 24. Like Bio-Rad's original ddPCR system released in 2011, Bio-Rad's ddSEQ system relies on droplet creation.

D. Proceedings Before The International Trade Commission

1. 10X Initiates The Investigation Below

On January 11, 2018, 10X alleged that Bio-Rad violated 19 U.S.C. § 1337 based upon Bio-Rad's importation of components of its ddSEQ system into the United States, and the ITC instituted this investigation. Appx00598.

2. ALJ Denies 10X's Motion For Summary Determination On Ownership, Recognizing Elements Of The Claims Were Conceived At QuantaLife/Bio-Rad

On February 21, 2019, the ALJ issued its order on summary determination

relating to Bio-Rad's claim that it owned a share of 10X's patents based on Saxonov's and Hindson's work at QuantaLife/Bio-Rad. Appx00673.

As set forth by the ALJ in her order, Bio-Rad identified "eleven claim elements in the asserted patents that were conceived by Saxonov and Hindson at QuantaLife prior to the Bio-Rad acquisition." Appx00677 (identifying technical elements).

The ALJ ruled that "10X does not dispute that Dr. Saxonov and Dr. Hindson conceived of certain of these ideas at QuantaLife" but that 10X argued conception was not "complete" until later. Appx00678. Although recognizing that at least "certain elements of these inventions were conceived while Saxonov and Hindson were employed at QuantaLife," the ALJ ruled "the inventions as a whole may not have been fully conceived until they left Bio-Rad to found 10X." *Id*. Accordingly, the ALJ set the matter for trial. *Id*.

3. Trial And Final Determination

On March 25-29, 2019, the ALJ held an evidentiary hearing.

On July 12, 2019, the ALJ issued the Initial Determination ("ID"), finding there was a violation with respect to certain claims of the '024, '468, and '530 Patents. Appx00139.[3] With respect to ownership, the ID ruled that the inventions

[3] 10X had asserted a fourth patent, No. 9,644,204. The ID found no infringement of this patent, which was affirmed by the Commission. Appx00065; Appx00071-

must be final before any ownership interest can attach: "[t]he only fact that matters is the actual time when the inventors conceived of the inventive idea embodied in the asserted patents." Appx00282.

After the parties filed petitions for review of the ID, on February 12, 2020, the Commission issued its final determination affirming infringement of the patents-in-suit, and issued a limited exclusion order and a cease and desist order against Bio-Rad. Appx00005; Appx00015; Appx00031, Appx00109-00121.

SUMMARY OF ARGUMENT

The Commission's final determination should be reversed on several independent grounds.

1. Bio-Rad owns a pro rata share of the 10X patents.

Across the three 10X patents, there are six named inventors. Two of the named inventors are former QuantaLife/Bio-Rad employees: Saxonov and Hindson. While at QuantaLife/Bio-Rad, they conceived of ideas reflected in the 10X patents. Bio-Rad is not asserting that it owns the contributions by the other co-inventors who may have made later contributions at 10X. The Commission's critical error was finding that a claimed invention had to be fully complete before ***any*** inventors' ownership interest could apply. This misreads the relevant contracts as well

00072. 10X has not appealed this finding, and consequently the '204 Patent is no longer at issue.

principles of co-inventorship—none of which requires a single "eureka" moment or a completed invention to trigger ownership rights. Indeed, under well-established principles of joint inventorship, each co-inventor does not have to conceive of the entire invention.

Moreover, the evidence goes beyond a partial contribution. The technical evidence ***admitted at trial*** demonstrates every critical limitation of the independent claims, from overall architecture to specific reagents, was conceived at QuantaLife/Bio-Rad: using droplets as partitions (all patents); having a reagent delivery system within each droplet (all patents); using oligonucleotides as barcodes (all patents); using gel beads as a delivery system (all patents); using a stimulus to release oligonucleotide barcodes ('024 Patent); using microfluidic channels to generate droplets, and having a device with double channel junctions ('468 Patent); isolating a single cell and barcode within a droplet ('530 Patent); and using the system for sample preparation for, *e.g.*, next generation sequencing (all patents).

Not only did the Commission err in its ownership analysis, but it erred in finding 10X had met its burden of proof of infringement and/or domestic industry for each of the three 10X patents.

2. This Court should reverse the finding of infringement with respect to the '024 Patent. This patent claim requires two separate objects "releasably attached" to each other: a gel bead and an oligonucleotide. The claim further

requires a "stimulus" be applied "to the gel bead." In Bio-Rad's products, it is undisputed that the accused stimulus is an enzyme complex that acts on the oligonucleotide, not the gel bead. Under the plain language of the claim, there can be no infringement. The Commission did not even address this argument, which was Bio-Rad's primary non-infringement position.

3. This Court should reverse the finding of infringement with respect to the '468 Patent. The invention requires the intersection of two aqueous streams—a stream containing the sample and another containing the barcodes—to form an "aqueous mixture" at a first junction on a microfluidic device, where the "aqueous mixture" then gets encapsulated in a droplet when it intersects an oil stream at a second junction. There is, however, no "aqueous mixture" in the accused products. If an "aqueous mixture" was formed before droplet formation, the accused products and processes would not work. In the words of 10X's expert, there would be a "big mess."

4. This Court should reverse the finding of a violation with respect to the '530 Patent. This method claim requires the sequential steps of first generating at least 1,000 droplets that each include a single bead with at least 1,000 barcodes, and a single cell with a plurality of polynucleotides; then subsequently detaching a plurality of barcodes and barcoding a plurality of polynucleotides in each of the at least 1,000 droplets. Similar to the issues underlying the '468 Patent dispute, all the

technical evidence demonstrates that the reactions occur as soon as the chemicals mix and that barcodes detach from the beads immediately when droplets are generated. Thus, 10X cannot and did not meet its burden of proving that a plurality of barcodes detach and barcode a plurality of polynucleotides in each of at the least 1,000 droplets, ***after*** all 1,000 droplets are created, as required by the last step of the '530 claims.

Indeed, the Commission acknowledged that the ID did not provide any evidence to support 10X's allegations that it practiced its own patents to meet the ITC's domestic industry requirement. To address that deficiency, the Commission erred in *sua sponte* relying on evidence from a 10X investment presentation the Commission found demonstrated that at least 1,000 droplets were still detaching barcodes after the last droplet was formed. Not only does the document not show this, but it is an investment presentation unconnected to the actual products at issue and which was never cited by any expert in support of a domestic industry analysis. Importantly, it contradicts documentation that 10X uses to describe its actual products to the scientific community, showing its beads dissolve immediately. The science demonstrates that neither Bio-Rad's accused products nor 10X's products practice the '530 Patent.

Finally, the claims of the '530 Patent are indefinite. The ALJ and Commission have issued at least four conflicting constructions over the course of the

Investigation, demonstrating the scope of the claimed invention cannot be determined with reasonable certainty by those skilled in the art. Bio-Rad and the public are entitled to know the scope of the claims, and this is not possible given the shifting constructions.

STANDARDS OF REVIEW

The Commission's findings regarding infringement are questions of fact reviewed for substantial evidence. *See, e.g.*, *Linear Tech Corp. v. ITC*, 566 F.3d 1049, 1060, 1063 (Fed. Cir. 2009) (reversing Commission). Legal determinations, however, including claim constructions and interpretations of contractual language, are reviewed *de novo*. *See* 19 U.S.C. § 1337(c); 5 U.S.C. § 706(2)(E); *Vizio, Inc. v. Int'l Trade Comm'n*, 605 F.3d 1330, 1336, 1340-41 (Fed. Cir. 2010) (reversing Commission's claim construction on *de novo* review); *The Medicines Co. v. Hospira, Inc.*, 881 F.3d 1347, 1350 (Fed. Cir. 2018) ("Contract interpretation is a question of law that we review de novo.").

Bio-Rad's claim of ownership rights is based on contract, and the standard for such an affirmative defense is preponderance of the evidence. *See, e.g.*, *Synopsys, Inc. v. Magma Design Automation, Inc.*, No. C-04-3923 MMC, 2007 WL 322353, at *18 (N.D. Cal. Jan. 31, 2007) (applying preponderance standard to patent ownership claim).

ARGUMENT

I. THE COMMISSION ERRED IN DENYING BIO-RAD'S CO-OWNERSHIP INTEREST IN THE PATENTS-IN-SUIT

The Commission applied the wrong legal standards and deprived Bio-Rad of its pro rata ownership interest in the patents-in-suit. Bio-Rad's interest is based upon work done by Saxonov and Hindson while employed by and under contract to Bio-Rad and its predecessor, QuantaLife. Saxonov and Hindson are named inventors on all three patents along with four others.

The Commission's critical error was to assume the contracts and law required complete conception at QuantaLife/Bio-Rad. Appx00108. That is incorrect. Co-inventors do not need to conceive the entirety of the final invention. Nor do the contracts require it.

A. Legal Standards

1. Bio-Rad's Property Rights Derive From Contracts

It is undisputed that Saxonov and Hindson were scientists at QuantaLife who executed contracts broadly assigning all their intellectual property, including all "ideas, processes . . . works, inventions, discoveries" conceived, developed, or created at QuantaLife "whether not patentable." Appx03199; Appx03209. Bio-Rad acquired QuantaLife and all its assets, and Saxonov and Hindson then executed similar contracts with Bio-Rad. Appx03193; Appx03195; Appx00260. California state law governs the contracts. Appx03202; Appx03212; Appx03194; Appx03196.

Akazawa v. Link New Tech. Int'l, Inc., 520 F.3d 1354, 1357 (Fed. Cir. 2008) ("state law, not federal law, typically governs patent ownership.").

2. Patents Can Be Divided And Co-owned

Patents can be assigned in part and co-owned by multiple assignees. 35 U.S.C. § 261 ("[P]atents shall have the attributes of personal property. . . . Applications for patent, patents, or any interest therein, shall be assignable in law by an instrument in writing."); *Vaupel Textilmaschinen KG v. Meccanica Euro Italia SPA*, 944 F.2d 870, 875 (Fed. Cir. 1991) ("[A patent] is, in effect, a bundle of rights which may be divided and assigned, or retained in whole or part").

3. Inventions Can Develop Over Time

This Court's precedent demonstrates that inventions do not have to occur in a single instant, *i.e.*, there is no requirement for a eureka moment. *See, e.g.*, *FilmTec Corp. v. Hydranautics*, 982 F.2d 1546, 1551-63 (Fed. Cir. 1992) (former employer of the named inventor had an ownership interest in the asserted patent based on the named inventor's partial contributions during employment, even though those contributions did not meet every limitation in even a single claim); *Israel Bio-Eng'g Project v. Amgen, Inc.*, 475 F.3d 1256, 1268 (Fed. Cir. 2007) (finding two companies both had a "pro rata undivided ownership interest" in a patent based on the four co-inventors' partial contributions over a period that spanned employment at both companies).

Likewise, in *Board of Trustees of Leland Stanford Junior University v. Roche Molecular Systems, Inc.*, 487 F. Supp. 2d 1099 (N.D. Cal. 2007), the court found that an inventor's work at his former employer was "sufficient to trigger the assignment provision" in his assignment agreement, even though "the patented invention was not ***completed*** until after [the inventor] left" the first employer. *Id.* at 1116-17, *aff'd,* 583 F.3d 832 (Fed. Cir. 2009), *aff'd,* 563 U.S. 776 (2011). The court found the first employer had an ownership interest in the patent even though the inventor's later work at the second employer "was 'crucial to the invention.'" *Id.*

As the above cases demonstrate, the case law is replete with examples of inventions being started at one company, and then completed at a second company—with broad contractual rights giving co-ownership interests in the resulting patents to the first company.

4. Under Principles Of Joint Inventorship, Each Co-Inventor Does Not Have To Conceive Of The Entire Invention

The Federal Circuit has endorsed looking to patent law "for guidance" in determining patent ownership. *See Am. Tel. & Tel. Co. v. Integrated Network Corp.*, 972 F.2d 1321, 1325 (Fed. Cir. 1992). The statutory standard for joint inventorship sets forth that individuals can be co-inventors on a patent claim even if "(1) they did not physically work together or at the same time, (2) each did not make the same type or amount of contribution, or (3) each did not make a contribution to the subject matter of every claim of the patent." 35 U.S.C. § 116.

Following the statute, the case law reflects that each joint inventor does not need to conceive of the entire invention:

> Each [joint inventor] needs to perform but a part of the task if an invention emerges from all of the steps taken together. It is not necessary that the entire invention concept should occur to each of the joint inventors, or that the two should physically work on the project together. ***One may take a step at one time, the other an approach at different times.***

Vanderbilt Univ. v. ICOS Corp., 601 F.3d 1297, 1302 (Fed. Cir. 2010) (quoting *Monsanto Co. v. Kamp*, 269 F. Supp. 818, 824 (D.D.C 1967)). A person's contributions to steps that are significant on the path to the final invention is enough to qualify that person as a co-inventor. *See, e.g.*, *In re VerHoef*, 888 F.3d 1362, 1366 (Fed. Cir. 2018) (contribution of a single idea entitles joint inventorship); *Pannu v. Iolab Corp.*, 155 F.3d 1344, 1351 (Fed. Cir. 1998) (same). Saxonov's and Hindson's extensive QuantaLife/Bio-Rad work established them as co-inventors even if they or other co-inventors contributed additional work later.

B. The Commission Legally Erred In Affirming The ID's Approach To Ownership

1. The ID Erred In Finding That Patents Reflect An "Inventive Concept" And That Ownership Requires Complete Conception Of Final Claims

With respect to elements of the claims, the ALJ confirmed that "10X does not dispute that Dr. Saxonov and Dr. Hindson conceived of certain of these ideas" while employed at QuantaLife and Bio-Rad. Appx00678. This should have ended the inquiry.

Ignoring that the claims had multiple elements and multiple inventors, however, the ID framed the ownership issue as a binary question involving a single idea reflected in the patented invention: "is there evidence that the idea [singular] embodied in the asserted patents was conceived by Drs. Hindson and Saxonov during the period in which they were employed by QuantaLife and Bio-Rad?" Appx00277. The ID explained:

- "[T]he only fact that matters is the actual time when the inventors conceived of the inventive idea embodied in the asserted patents." Appx00282.
- "[T]he inventive idea is a specific arrangement of elements which, when combined, works to achieve a desired goal." Appx00283.
- Bio-Rad only owns an interest if "Drs. Hindson and Saxonov actually conceived the inventive idea embodied in the asserted patents during the employment period." Appx00286.

The upshot of the ID's erroneous formulation is that the "idea" must be the final, complete set of claimed elements, arranged in the final order. Appx00283. However, neither the contracts nor patent law require a final complete invention before ownership rights attach for a co-inventor's work.

2. The Commission Affirmed The ID's Error

The Commission affirmed the ID's erroneous approach, holding that Bio-Rad had to prove a singular "inventive concept" while Saxonov and Hindson were at QuantaLife/Bio-Rad—*i.e.*, "the specific arrangement of elements claimed in the asserted patents." Appx00108; *see also id.* ("[T]he inventive concept is the

combination and specific arrangement of elements laid out in the claims of the asserted patents."); *id.* (affirming the ID's reasoning that "the inventive idea is a specific arrangement of elements which, when combined, works to achieve a desired goal"). This was error.

3. The Commission's Opinion Contradicts Settled Law

(a) The Contracts Do Not Require A Completed Invention

Hindson's and Saxonov's agreements are directed to a broad definition of intellectual property: "any and all ***ideas***, ***processes***, . . . ***works, inventions, discoveries,*** . . . and improvements or enhancements to any of the foregoing . . . . ***whether or not patentable***." Appx03199; Appx03209. This language does not require a complete invention.

First, under California law, "[t]he language of a contract is to govern its interpretation, if the language is clear and explicit, and does not involve an absurdity." Cal. Civ. Code § 1638; *see also, e.g.*, *Buckhorn Inc. v. ORBIS Corp.*, 547 F. App'x 967, 972 (Fed. Cir. 2013) (quoting Cal. Civ. Code § 1638) (reversing district court).

Here, the plain language in the agreements provide for ownership of "ideas" and intellectual property "whether or not patentable." Appx03199; Appx03209. The Commission was wrong to import a requirement that the ideas must be "the specific arrangement of elements claimed in the asserted patents." Appx00108.

Second, "[t]he words of a contract are to be understood in their ordinary and popular sense, rather than according to their strict legal meaning; unless used by the parties in a technical sense." Cal. Civ. Code § 1644. Indeed, the Federal Circuit has similarly concluded that employment contracts assigning "inventions" "conceived" by employees are not necessarily bound by how those terms are used in patent law. *See Am. Tel. & Tel. Co.*, 972 F.2d at 1324 ("We disagree with the district court that conception of inventions, as used in the employment agreement, is solely a technical question of patent law.").

Numerous courts have similarly found that contracts can assign intellectual property and "inventions" "conceived" by employees in a manner broader than inventions recognized by federal patent law. *See, e.g.*, *Venclose Inc. v. Covidien Holding, Inc.*, No. 16-cv-07372-EJD, 2017 WL 3335984, at *7 (N.D. Cal. Aug. 4, 2017) (agreement covered inventions defined as something broader than inventions recognized by patent law); *Motorola, Inc. v. Lemko Corp.*, No. 08 C 5427, 2012 WL 74319, at *12 (N.D. Ill. Jan. 10, 2012) (the "broad language" of inventors' employment agreements "specifically reference 'ideas,' not just 'inventions'").

Here, it is apparent from the plain language of the agreements that they are written—and thus convey the parties' intent—to broadly capture the full scope Saxonov's and Hindson's work at QuantaLife/Bio-Rad in order to protect the investment the company made in research and development. Appx03199;

Appx03209. The Commission's imposition of a requirement of complete, final ordering of all elements by all inventors contradicts the terms of the contracts and violates the express intent of the parties to the contracts that assignments should not be limited to completed, patentable inventions. Cal. Civ. Code §§ 1638, 1644; *Am. Tel. & Tel. Co.*, 972 F.2d at 1324.

4. Patent Law Does Not Require A Single Moment Of "Inventive Conception" Or That All Co-Inventors Conceive All Elements Of A Patent Claim

The Commission erred in ignoring principles of joint inventorship that a person can be a co-inventor even if the invention is not complete. The ID (affirmed by the Commission) wrongly required Bio-Rad to establish "the actual time when the inventors conceived of the inventive idea embodied in the asserted patents." Appx00282. It is well-settled, however, that inventions can develop over time.

The Patent Act contemplates that an invention can be jointly made, and explicitly provides that each co-inventor need "not make the same type or amount of contribution" and need not be working "at the same time." 35 U.S.C. § 116. Indeed, joint inventions can develop over time with the final result emerging from "all of the steps taken together"; one co-inventor "may take a step at one time, the other an approach at different times." *Vanderbilt,* 601 F.3d at 1302 (quoting *Monsanto*, 269 F.Supp. at 824). Each person contributing a significant step towards the final result is a co-inventor, and each co-inventor does not have to conceive of

the entire invention. *Id*. at 1303.

It is undisputed the 10X patents were jointly invented by the multiple co-inventors named on each of the patents; Hindson and Saxonov are two of six total. Appx00346 (listing three co-inventors); Appx00377 (listing three co-inventors); Appx00408 (listing six co-inventors). Each joint inventor has rights to the whole patent, even if a co-inventor contributed to only part of one claim. *See, e.g.*, *Ethicon, Inc. v. U.S. Surgical Corp.*, 135 F.3d 1456, 1465 (Fed. Cir. 1998).

As the ALJ recognized, it is largely undisputed that Saxonov and Hindson conducted technical work at QuantaLife/Bio-Rad directed to using barcodes and droplets for next generation sequencing. That work (without considering any further work they may have done at 10X) qualifies them as co-inventors—which demonstrates QuantaLife/Bio-Rad owns a share of the patents.

C. The Facts Show Saxonov and Hindson Conceived Of Significant Ideas Reflected In The 10X Patent Claims

Saxonov and Hindson are joint inventors of the 10X patents based on their work at QuantaLife/Bio-Rad, even if all of the work was not completed until later. "[T]o be a joint inventor, an individual must make a contribution to the conception of the claimed invention that is not insignificant in quality, when that contribution is measured against the dimension of the full invention." *Fina Oil & Chem. Co. v. Ewen*, 123 F.3d 1466, 1473 (Fed. Cir. 1997). Here, Saxonov's and Hindson's work at QuantaLife/Bio-Rad meet that threshold.

1. Saxonov and Hindson Conceived Of Key Aspects Of The Claimed Inventions, If Not The Entirety Of The Claims, At QuantaLife/Bio-Rad

The basic documentary facts of Saxonov's and Hindson's work at QuantaLife and Bio-Rad are undisputed. In summary, Hindson and Saxonov developed a four-part system for single cell sequencing while at QuantaLife/Bio-Rad, involving: (1) partitioning sample into droplets, (2) creating a reagent delivery system, (3) combining the sample and reagent delivery system within droplets using microfluidics, and (4) tracking the sample-reagent reaction complex with a barcode mechanism. *See, e.g.*, Appx10045 at 176:12-177:1.

(a) All Asserted 10X Patents Are Directed To Solutions To Next Generation Sequencing, Which Was Hindson's Idea At QuantaLife

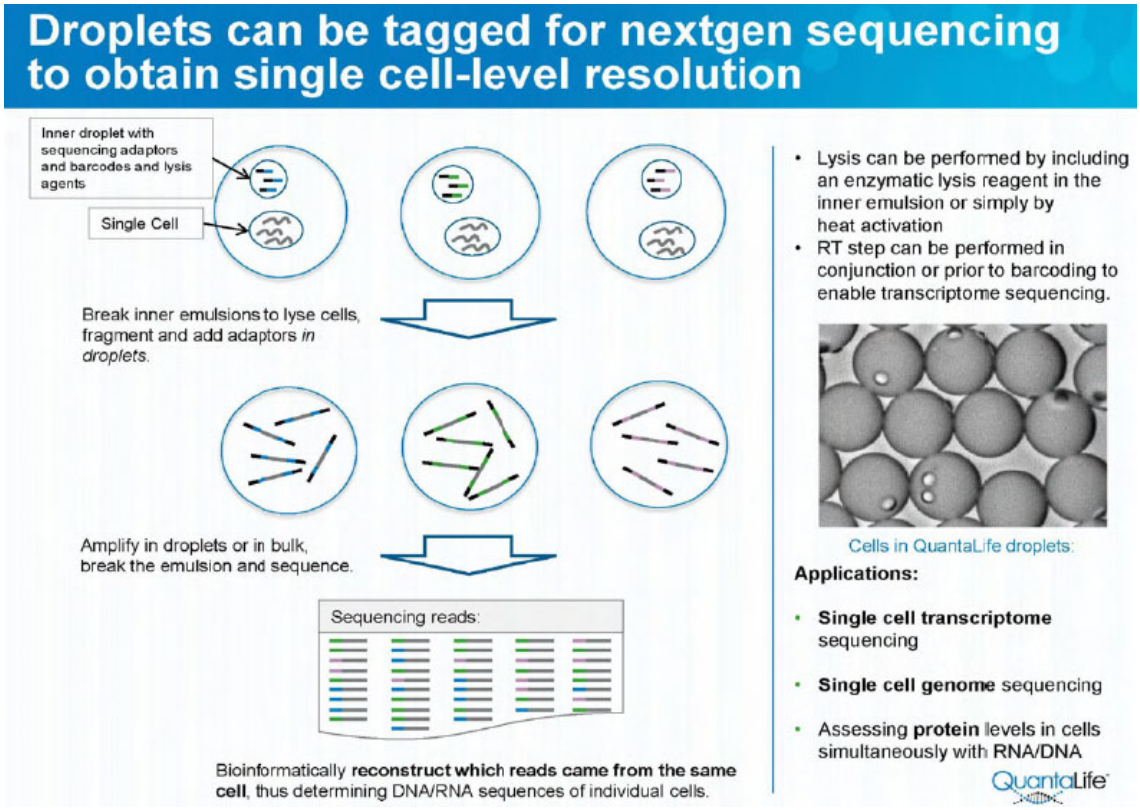
There is no dispute that each of the three 10X patents are directed to an approach to prepare samples for next generation DNA sequencing—e.g., barcoding the polynucleotides from the single cell that is partitioned in a droplet so that the sequence from those polynucleotides can be traced back to a particular individual cell. 10X touts these features repeatedly. *See, e.g.*, Appx10045 at 176:6-177:1.

But this architecture and solution was already developed at QuantaLife. For example, at QuantaLife, Hindson presented his concept for a reagent delivery system, describing that "[d]roplets can be tagged for nextgen sequencing to obtain single cell-level resolution." Appx03441-03442; Appx10040 157:7-23.

(b) The 10X Patents Are Directed To Tracking The Sample-Reagent Reaction Complex, Which Hindson Developed At QuantaLife

Each of the patents requires being able to track the reagent-sample reaction complex with a barcode. Appx00375 at 33:55-34:7; Appx00406 at 33:55-34:9; Appx00450-00451 at 47:58-49:4.

Hindson developed this idea at QuantaLife, as demonstrated in Hindson's presentation describing the delivery of oligonucleotide barcodes within droplets to the sample cell in the droplet. Appx03441-03442; Appx10041 at 160:17-161:21. Below is Hindson's image of how to tag and track the complex:



Appx03442. This is a significant piece of evidence because it lays out Hindson's ideas for a single cell system, which he later sought to patent at 10X. The slide may not reflect all the elements of the claim, but compared to each claim as a whole, it reflects "a contribution" certainly "not insignificant" in quality. *Fina*, 123 F.3d at 1473.

The Commission downplayed the slide and Hindson's related work as being "droplet-in-droplet architecture" and therefore different from the 10X Patents. Appx00105. Not so. Hindson developed the overall architecture for the 10X solution at QuantaLife, and that includes using droplets, tagging them with barcodes, and obtaining "single cell-level resolution" for DNA sequencing. Appx03442. These are specific design choices that are reflected in the 10X patents.

Although the law sets "no explicit lower limit on the quantum or quality of inventive contribution required for a person to quality as a joint inventor," *Fina*, 123 F.3d at 1473, what Hindson developed at QuantaLife surely meets the threshold based on this slide alone: it addresses the problem with a specific solution and architecture.

And, indeed, this slide is not the only piece of evidence; Hindson's trial testimony confirmed the slide reflects the same architecture claimed in the 10X patents. Appx03442; Appx10041 at 160:5-161:6. As Hindson testified, the idea shown on this slide is to deliver the reagents and oligonucleotide barcodes to the

sample. Appx10041 at 161:7-10. The "inner droplet" contains multiple identical barcodes that are unique to each inner droplet. Appx03441-03442. The next step, as depicted on the slide, is to "[a]mplify" the barcoded sample, then break the outer droplets to pool all the barcoded sample together, then sequence. Appx03441-03442; Appx10041 at 161:11-13. The result, as shown on the bottom of the slide is one can "[b]ioinformatically reconstruct which reads came from the same cell, thus determining DNA/RNA sequences of individual cells"—*i.e.*, "obtain[ing] single cell-level resolution." Appx03441-03442; Appx10041 at 161:22-162:2; Appx10199 at 779:5-780:23.

The amplification step shown on these slides is also found in claims of the 10X patents. *See, e.g.*, Appx00375 at 33:55-34:7 ('024 Patent, Claim 1); Appx00406 at 34:35-36 ('468 Patent, Claim 11); Appx00451 at 49:23-26 ('530 Patent, Claim 7).

(c) All Asserted 10X Patents Use Barcoding To Tag Sample DNA, Which Saxonov And Hindson Developed At QuantaLife

Each asserted claim requires barcodes to tag polynucleotides. Appx00375 at 33:55-34:7; Appx00406 at 33:55-34:9; Appx00450-00451 at 47:58-49:4. To further their ideas for single cell analysis, Saxonov and Hindson referred to their "partition barcoding scheme" from QuantaLife.

For example, on April 14, 2011, while at QuantaLife, Saxonov sent a detailed technical email to Hindson describing ideas for next generation sequencing.

Appx02907. In this email, titled "idea for sample partitioning and barcode tagging for sequencing," Saxonov described what he called "[o]ur partition-barcoding scheme." Appx02910; Appx10198 at 775:11-18. He also conceived that these ideas can be used to analyze sequencing read data "to determine which transcripts came from the same cell. This way the massive capacity of nextgen sequencing can be applied to large collections of cells while preserving single cell resolution." Appx02912; Appx10198 at 777:6-10.

In other words, this "partition-barcoding scheme" Saxonov and Hindson conceived of at QuantaLife could be used to preserve cell-level information in next generation sequencing, and one "could apply . . . the capacity [of] next-gen sequencing to large collections of cells while preserving single cell resolution." Appx10198 at 776:22-777:5.

(d) All Asserted 10X Patents Use Aqueous Droplets In Oil To Partition Sample For Sequencing, Which Hindson Conceived At QuantaLife

The claims of each of the three patents require droplets to partition sample. Appx00375 at 33:55-34:7 ('024 Patent, Claim 1); Appx00406 at 33:55-34:9 ('468 Patent, Claim 1); Appx00450-00451 at 47:58-49:4 ('530 Patent, Claim 1). Hindson developed this approach at QuantaLife. As confirmed at the hearing, there are many potential ways to partition sample for analysis, but Hindson's idea at QuantaLife was to use ***droplets***. Appx10036 at 138:19-25. The use of droplets to partition

sample (and achieve a single cell per partition) is fundamental to 10X's claimed inventions, and Hinson's choice to focus on this method is a significant aspect of the inventions he developed while at QuantaLife.

(e) The 10X Patents Claim Combining Sample With Reagent Delivery System Within Droplets, Which Hindson Developed At QuantaLife

Another fundamental aspect of the claimed inventions in the 10X patents is the idea of combining sample and reagent within a droplet so that the reagent can react with the partitioned sample —*i.e.*, separated—from other sample and reagents. Appx00375 at 33:55-34:7 ('024 Patent, Claim 1); Appx00406 at 33:55-34:9 ('468 Patent, Claim 1); Appx00450-00451 at 47:58-49:4 ('530 Patent, Claim 1). Hindson conceived of this idea at QuantaLife.

For instance, Hindson conceived of a "reagent delivery system" to deliver reagents within droplets. Appx02781; Appx02783; Appx10037 at 142:5-146:7. As recorded in his lab notebook, the system involved a preprocessing step using fluorescent beads as "barcodes." Appx10037 at 144:7-12; Appx02781. The inner droplets containing the reagents are then delivered to a larger droplet containing sample using microfluidic channels. Appx10037 at 144:18-145:8; Appx02781. The next step in this system involved using a stimulus to release the "inner" reagents but not the "outer" emulsion, which allows the contents to mix within the droplet. Appx02783; Appx10037-10038 at 145:9-146:7. The fundamental idea of having

sample and a reagent delivery system with a barcode within a droplet is the basis of all the 10X patent claims.

(f) The 10X Patents All Claim Oligonucleotides As Barcodes, Which Hindson And Saxonov Developed At QuantaLife

The three 10X patents all claim using oligonucleotides as the barcode to tag the sample. Appx00375 at 33:59-60 ('024 Patent, Claim 1); Appx00406 at 33:57-58 ('468 Patent, Claim 1); Appx00442 at 31:28 ('530 Patent).

Hindson and Saxonov conceived of the idea to use oligonucleotides as barcodes within droplets at QuantaLife. Appx02911-02912 (using "partition-specific barcoded primers" for single cell transcriptome sequencing); Appx03075-03076 at 95:23-96:1 (testifying, with respect to Appx02907-Appx02913, that "a barcoded primer can be or can include an oligonucleotide"). This QuantaLife/Bio-Rad idea was specifically incorporated into the 10X patent claims.

(g) The Idea To Use Gel Beads As Part Of The Delivery System Was Conceived At QuantaLife—Based On The Evidence Admitted At Trial

There is no dispute about the preceding technical information that applies to all patents. Based on that evidence alone, the ideas Hindson and Saxonov conceived at QuantaLife/Bio-Rad were enough to trigger their contracts—thus providing co-ownership rights in the 10X patents to Bio-Rad.

There is an additional element that is present in all the patent claims, and that is use of gel beads as part of the reagent delivery system. Appx00375 at 33:55-34:7 ('024 Patent, Claim 1); Appx00406 at 33:55-34:9 ('468 Patent, Claim 1); Appx00450-00451 at 47:58-49:4 ('530 Patent, Claim 1). The ID (and consequently Commission Opinion) erred in not finding that a preponderance of the evidence established that Hindson conceived of this element as well at QuantaLife. Although there were pretrial evidentiary objections about gel beads as part of this litigation, the ***evidence admitted at trial*** demonstrated that, at a minimum, the genesis of the idea for using gel beads was at QuantaLife.

First, there is no dispute that Hindson and Saxonov were actively collaborating about solutions for next generation sequencing. Appx02904; Appx03441-03442. In that context, on April 14, 2011, Saxonov sent a detailed email to Hindson and another QuantaLife employee about the idea of using droplets as part of a "partition-barcoding scheme" for single cell sequencing. Appx02907.

On April 14, 2011, the same day that Saxonov sent his "partition-barcoding scheme" email, Hindson emailed Saxonov a link to a peer-reviewed article on gel beads by Harvard's Jeremy Agresti and others, referred to as the "Beating Poisson" paper. Appx10043-10044 at 169:18-171:23. The "Beating Poisson" paper discloses using microfluidics to deliver deformable gel beads to droplets and teaches that the

gel beads can be functionalized with DNA. Appx02683-02685; Appx10043 at 166:25-167:14; 168:4-24.

On April 15, 2011, the next day, Saxonov sent a follow-on email to his "partition-barcoding scheme" email to say: "another thought related to this—coming out of talking to Ben [Hindson]. . . . Ben suggested beads." Appx02303.

Significantly, in pretrial corporate testimony, Hindson testified that, of all the named inventors, he was the one who had the idea to use porous gel beads in the 10X Patents. Appx02930 at 47:6-12. Hindson confirmed at the hearing that he learned about porous gel beads from the 2009 "Beating Poisson" article. Appx10043-10044 at 166:10-14, 170:24-171:7; Appx02935-02936 at 53:4-54:9, 54:15-23. All of the preceding evidence was admitted at trial.

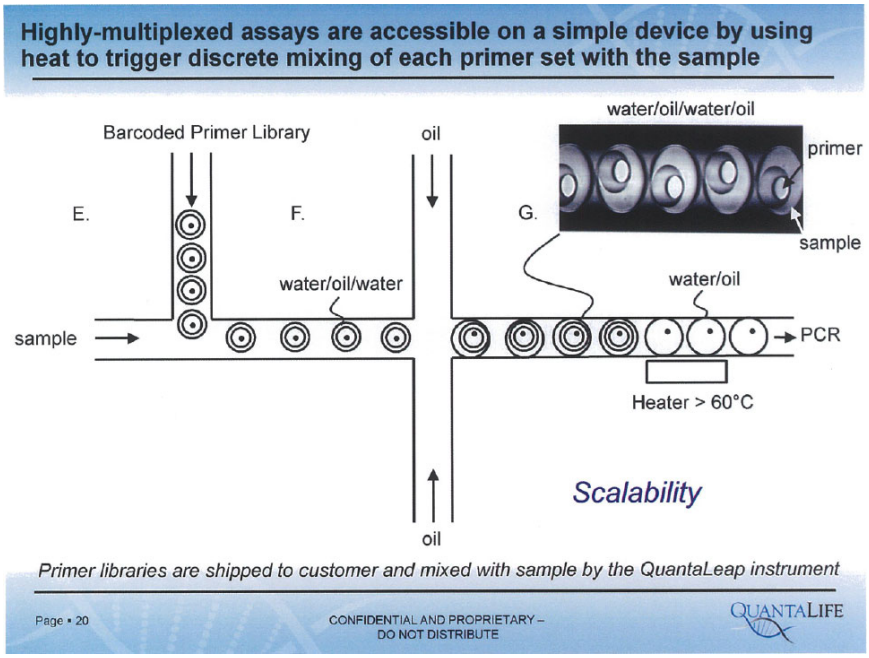
Thus, the preponderance of the evidence shows that Hindson conceived of the ***idea*** for using gel beads as the delivery reagent at QuantaLife.[4]

(h) The '468 Patent Is Specifically Directed To Double Junction Microfluidics To Combine Sample And Reagent, Which Hindson Developed At QuantaLife

[4] The ID credited Hindson's testimony at the hearing that he "did not remember" seeing the gel bead article at QuantaLife. Appx00289. However, what Hindson "remembered" in 2019 is not relevant. The contemporaneous documentary evidence from 2011 shows that Hindson had possession of the article and emailed it to Saxonov on the very day that Saxonov circulated his idea for oligonucleotide barcodes in droplets, which Saxonov followed up on the next day by emailing that "Ben suggested beads." Appx02303. The documents speak for themselves that the ideas were in hand at QuantaLife.

Not only were fundamental aspects of all claims developed at QuantaLife, but also specific elements of individual claims as well.

Claim 1 of the '468 Patent is directed to a "microfluidic device" that includes a "first junction" to form an aqueous mixture of sample and barcodes, and a "second junction" to generate droplets by capturing droplets of the mixture within an immiscible phase. Appx00406 at 33:55-34:9. Hindson conceived of this exact form of microfluidic device, with channels to form two junctions, at QuantaLife:



Appx02904; Appx10038 at 147:18-148:20. As can be seen, the microfluidic device contains a double junction exactly as claimed in the '468 Patent.

(i) The '024 And '468 Patents Are Directed To Providing At Least One Million Barcodes, Which Saxonov Developed At QuantaLife

The ’024 Patent claims a porous gel bead “wherein said porous gel bead comprises at least 1,000,000 oligonucleotide molecules.” Appx00375 at 33:58-60. The ’468 Patent also claims “providing at least 1,000,000 oligonucleotide molecules comprising barcode sequences.” Appx00406 at 33:57-58. Saxonov developed this specific idea at QuantaLife. Appx02908 (conceiving of using one million different partitions, where “[e]very one of the million partitions ends up with its own unique combination of barcodes”); Appx03070 at 90:2-11 (explaining that “[e]ach partition would have its own unique combination of sequences that were different from the combinations in other partitions,” and that “every combination in a partition would be a combination of barcode sequences”).

* * *

In sum, the preceding evidence of record demonstrates that Hindson and Saxonov worked together at QuantaLife/Bio-Rad to develop specific ideas for the architecture now claimed in the 10X patents, and specific ideas to use microfluidics, droplets, barcodes, gel beads, and other reagents to isolate single cells for next generation sequencing. This overwhelming technical evidence qualifies them as co-inventors based on that work and triggered their assignment contracts—***even if*** all the elements were not complete at QuantaLife/Bio-Rad. *Vanderbilt,* 601 F.3d at 1302.

Bio-Rad should have been awarded a pro rata share of the 10X patents.

D. The Commission Introduced Additional Errors In Affirming The ID Below

Not only did the Commission wrongly affirm the ALJ's legal finding that the inventions must be completed at QuantaLife/Bio-Rad, but the Commission also introduced additional errors to support its conclusion.

1. The Commission Wrongly Found That Bio-Rad Limited Its Ownership Defense To The '468 Patent

At trial, Bio-Rad presented evidence of ownership relating to all the 10X patents. The ID (as described above) erroneously found otherwise. In its opinion affirming the ID, however, the Commission stated because Bio-Rad had used claim 1 of the '468 Patent as a "representative" claim in answering the Commission's supplemental questions, "Bio-Rad's showing of ownership under its theory would be limited to the '468 Patent." Appx00104. This was error.

Bio-Rad argued that its ownership defense applied to all 10X patents, and appealed the ID's finding on these grounds. There are common claim elements across all the patents—and Bio-Rad consistently pointed that out to the ALJ and Commission. *See, e.g.*, Appx00677-00678; Appx00290-00292; Appx00806-00816.

After Bio-Rad appealed the ID's decision for all 10X patents, the Commission issued a notice of its determination to review the ID in part. Appx00828. The Commission also requested briefing from the parties "on certain issues under review" within strict page and time limits, and listed various questions, including

co-inventorship. Appx00828-00832. The Commission explicitly instructed "[t]he parties not to brief other issues on review, which are adequately presented in the parties' existing filings." Appx00831. Bio-Rad timely submitted its answers to the Commission's various questions. Appx00034.

The Commission's finding that Bio-Rad appealed only the '468 Patent from the ID is incorrect as a matter of record. In its petition for review of the ID, Bio-Rad addressed each of 10X's patents. Appx00806-00816. Indeed, in answering the Commission's supplemental questions, Bio-Rad stated: "As discussed herein and in Bio-Rad's petition for review, the Commission should find no violation as to any of the Asserted Patents." Appx00845.

Bio-Rad's briefing to answer the Commission's supplemental questions should not be seen as a limitation on the previous briefing, and no other party to the investigation was limited to just the answers they gave to the Commission's supplemental questions. The Commission gave no notice to Bio-Rad that the supplemental briefing would somehow be limiting. And, in any event, Bio-Rad incorporated by reference its arguments in the petition for review, thus preserving its arguments as to all patents. Appx00845; Appx00806-00816.

2. The Commission Wrongly Relied On Saxonov's '059 Patent To Counter Bio-Rad's Ownership Claim

In affirming the ID's ownership decision, the Commission stated that Bio-Rad is not entitled to Hindson's and Saxonov's share of the 10X patents because Bio-

Rad already has ownership of Saxonov's U.S. Patent No. 9,347,059. Appx00106. That is a false equivalence and not a reason to deny Bio-Rad's contract rights.

First, the contracts should be evaluated on their face. They are broad and cover all "IP." Appx03199; Appx03209. Bio-Rad owns a share of the 10X patents whether or not there is subject matter overlap with a patent Bio-Rad already owns.

Second, the '059 Patent lists only Serge Saxonov as an inventor. Appx02111. Hindson's contributions are separate and not reflected in the '059 Patent. Indeed, although Hindson collaborated with Saxonov at QuantaLife/Bio-Rad, he independently conceived of many ideas that appear in the 10X patent, such as, for example, the double junction microfluidic device, the reagent delivery system, and use of gel beads. *See supra* Section I.C.

Finally, when asked about his contributions to the 10X Patents, Saxonov pointed to the numerical limitations in the asserted patents such as the specific number of droplets, barcodes, and cells to claim. *See, e.g.*, Appx03161-03163 at 202:15-204:20 ("I'd say I contributed to the numbers."), Appx03170-03171 at 212:16-213:1. Since Saxonov testified that he actually came up with the number of barcodes to use while he was at Bio-Rad, this by itself demonstrates that it is Bio-Rad, not 10X, that is entitled to the co-inventor portion of the 10X asserted patents that Saxonov assigned to 10X.

3. The Commission Wrongly Excluded Evidence The ALJ Admitted At Trial

As described above, *supra* Section I.C.1(g), the evidence admitted at trial demonstrated the idea for using porous gel beads as claimed in the 10X patents arose at QuantaLife/Bio-Rad.

In affirming the ID's conclusion, however, the Commission pointed to a pretrial order limiting Bio-Rad's ability to introduce evidence at trial concerning gel beads. Appx00104. This was error. The ID itself considered the porous gel bead evidence and made no mention of the pretrial order. The Commission cannot revisit the trial record and make objections that the ALJ never made or sustained. Indeed, it is not surprising that the ALJ's ID did not mention the pretrial order because Bio-Rad complied precisely with it.

The ALJ's pretrial order expressly allowed Bio-Rad to "rely on certain documents to question Dr. Hindson's credibility" and found that "it may be appropriate to use Dr. Hindson's e-mails in a cross-examination if these issues are within the scope of a direct or re-direct examination." Appx00701. This is exactly what happened during the hearing before the ALJ. On direct examination, Hindson testified how he allegedly came up with the concept to use porous gel beads only after he left Bio-Rad. Appx01215-01217, Appx01221-01222. On cross-examination, counsel for Bio-Rad undermined this testimony and pointed to evidence that Hindson had the idea to use porous gel beads while he was at QuantaLife. Appx10042-10045 at 164:4-175:19. 10X did not object to any of this

examination at the hearing—nor could it, because this testimony was properly within the scope of the ALJ's order. *Id.* The ALJ allowed the testimony, and it was never excluded by the judge. Appx00701.

In fact, the ALJ cited to this testimony in her ID, and did not find that her pretrial ruling prohibited its consideration. Appx00288-00290. It was error for the Commission to reach back and apply a pretrial order to exclude this evidence after it was admitted.

In any event, even if evidence of the porous gel beads being conceived at QuantaLife were not considered, the copious undisputed evidence of Hindson and Saxonov working on droplet-based single cell analysis is enough to meet Bio-Rad's burden of proving by a preponderance of the evidence that it is entitled to a pro rata share of the 10X patents.

II. THE COMMISSION ERRED IN DETERMINING THAT BIO-RAD INFRINGED THE '024 PATENT

Bio-Rad does not infringe the '024 Patent. *See, e.g.*, *Southwall Techs., Inc. v. Cardinal IG Co*., 54 F.3d 1570, 1575 (Fed. Cir. 1995) ("[E]very limitation set forth in a claim must be found in an accused product, exactly."). In particular, the accused "stimulus" is not applied "to [the] porous gel bead" to release the oligonucleotide as the claims require; instead, the accused "stimulus" is an enzyme that destroys part of the oligonucleotide. Bio-Rad's approach is fundamentally different than what the claims require.

A. The '024 Patent Claims Require The Stimulus Be Applied To The Porous Gel Bead

The '024 Patent claims set forth, in part, a droplet that has a porous gel bead with at least 1,000,000 oligonucleotide molecules comprising barcode sequences, that are releasably attached to said porous gel bead. Appx00375 at 33:55-34:7. The claimed method requires "applying a stimulus ***to said porous gel bead*** to release said oligonucleotide molecules from said porous gel bead into said droplet." *Id.* at 33:65-67. There cannot be infringement if a stimulus is applied ***to the oligonucleotide*** and not the gel bead. To find otherwise thwarts the plain language and structure of the claim.

B. In Bio-Rad's System, The Accused Stimulus Is An Enzyme Complex Applied To The Oligonucleotide

In Bio-Rad's accused products, the gel bead is inert, and the stimulus is applied to the oligonucleotide. Appx03235, Appx03240-03251; Appx10088 at 345:13-25. As the ALJ found, the accused stimulus in Bio-Rad's product is an enzyme complex. Appx00164 ("no dispute" that enzyme "acts on the oligonucleotide"); Appx00037; Appx00059. The enzyme complex removes [redacted] within the oligonucleotide and also breaks the backbone of the oligonucleotide at that point. Appx00164-00165; Appx03223-03225, Appx03228, Appx03249-03250. Despite this undisputed evidence, the ALJ erroneously found that the stimulus was applied to the gel bead.

C. The Commission Erred In Affirming The ID

1. The ID Wrongly Treated The Gel Bead And Oligonucleotide As The Same Object When They Are Separate Objects

The Commission did not address Bio-Rad's primary non-infringement argument and simply affirmed the erroneous reasoning in the ALJ's ID.

The ALJ recognized that the accused "stimulus" acts on the oligonucleotide. Appx00164. But the ALJ found infringement because, according to the ALJ, "***the oligonucleotides are part of the gel bead [and a]ny stimulus applied to the oligonucleotide is therefore also applied to the gel bead***." Appx00165.[5] This was error.

The oligonucleotides are not "part of" the gel bead. Two objects that are attached to each other are distinct from one another. *See, e.g.*, *In re Cuozzo Speed Techs., LLC*, 793 F.3d 1268, 1280 (Fed. Cir. 2015), (in a claim with a speedometer "integrally attached" to a colored display, "attached" must be given meaning and the speedometer and colored display are "separate parts physically joined together"). In other words, in *Cuozzo*, the speedometer and display are not the same thing; one part is "attached" to the other. *Id*.; *Regents of the Univ. of Minn. v. AGA Medical Corp*., 717 F.3d 929, 939 (Fed. Cir. 2013) ("When a physical object is described as having been 'affixed,' 'joined,' 'connected,' or 'conjoined,' to another object, it means that

[5] The Commission determined not to review this portion of the ID, adopting the ID's reasoning. Appx00037.

those objects were previously physically separate.") (alterations adopted). Similarly, in the '024 Patent, even though the oligonucleotide is "attached" to the porous gel bead, they are separate objects.

In appealing the ID's erroneous finding that the two objects are the same for purposes of applying the stimulus, Bio-Rad cited well-settled authority to the contrary.[6] The Commission did not address any of this black letter law.

Underscoring how the oligonucleotides and gel beads are separate objects, the ALJ's claim construction provides that "releasably attached" means attached "in a manner that allows the ***attached object*** to be released." Appx00664. The "attached object" is the oligonucleotide. It is of no moment, as the ALJ found, that the attached oligonucleotides may be "inside the volume" of the porous gel beads. Appx00165 (citation omitted). The objects are separate but attached, like the disks in *Regents* and the speedometer and display in *Cuozzo*.

Furthermore, porous gel beads and oligonucleotides are entirely different materials. Oligonucleotides are not porous beads; they are made of nucleic acids.

---

[6] *See, e.g.*, *Cutsforth, Inc. v. Motivepower, Inc.*, 643 F. App'x. 1008, 1012 (Fed. Cir. 2016) ("coupled" items are separate); *ICHL, LLC v. Sony Electronics, Inc.*, 455 F. App'x. 978, 981 (Fed. Cir. 2011) ("When one structure is bonded to a second structure, those structures are plainly separate."); *Becton, Dickinson and Co. v. Tyco Healthcare Group, LP*, 616 F.3d 1249, 1255 (Fed. Cir. 2010) ("connected" items are "separate structures"); *Dorel Juvenile Group Inc. v. Graco Children's Products, Inc.*, 429 F.3d 1043, 1045 (Fed. Cir. 2005) ("removably secured" items are separate structures).

Appx10105 at 413:8-12 (10X's expert testifying oligonucleotide molecules are not gels). It is illogical for the oligonucleotide to be part of the gel bead for purposes of applying a stimulus, but separate for purposes of being attached and released. *See, e.g.*, *Cuozzo,* 793 F.3d at 1280 ("[I]t would 'be illogical to regard one unit to be 'attached' to itself.'"). The '024 Patent claim requires the stimulus to be applied to one of these two objects—the porous gel bead—and not the other.

By affirming that the oligonucleotide is "part of" the gel bead for purposes of applying the stimulus, the Commission rewrote the plain language of the claims and added an implied construction that does not exist, which is legal error. *See, e.g.*, *Vizio, Inc. v. Int'l Trade Comm'n*, 605 F.3d 1330, 1340-42 (Fed. Cir. 2010).

2. The Transitional Phrase "Comprising" Does Not Eliminate The Separateness Of The Physical Objects

The definitive claim language, by specifying two different objects with one "releasably attached" to the other, should end the inquiry. However, the ALJ relied on the term "comprising" to rule that the oligonucleotide could be part of the gel bead. Appx00165. This was error.

First, to read "comprising" in this manner eliminates the claim language that one object is "releasably attached" to another. Appx00375 at 33:61. "Comprising" does not serve to alter claim language. *See, e.g.*, *Dippin' Dots, Inc. v. Mosey*, 476 F.3d 1337, 1343 (Fed. Cir. 2007) ("comprising" cannot "abrogate claim limitations").

Second, the common transitional term "comprising" is used throughout the claims, for instance, "a droplet comprising a porous gel bead . . . ." Appx00375 at 33:57. But it is clear that the droplet and the gel bead are separate physical objects, and "comprising" does not eliminate their separateness. Likewise, having the gel bead comprise releasably attached oligonucleotides does not eliminate the separate physical nature of these two objects. *See, e.g.*, MPEP § 2111.03 (2018) ("The transitional term 'comprising,' which is synonymous with 'including,' 'containing,' or 'characterized by' is inclusive and open ended."); *Cuozzo*, 793 F.3d at 1271 (claim provided for a "speed limit indicator comprising . . . a speedometer integrally attached to said colored display"). It was error for the ID to rely on "comprising" to conflate two separate objects.

3. The Specification, 10X's Products, And Hindson's And Saxonov's Prior Work At QuantaLife/Bio-Rad All Involve Degrading The Bead, Not Enzymatically Cleaving The Oligonucleotide

The difference between the 10X claim language and the Bio-Rad system is not a word game. It is a fundamentally different approach to apply a stimulus to the gel bead versus the oligonucleotide.

The '024 Patent specification describes applying a stimulus to the gel bead to degrade the gel bead, thus releasing the oligonucleotides. Indeed, the specification

provides that the gel bead (referred to interchangeably as a "microcapsule"[7]) is "degradable upon the application of a stimulus to the microcapsule." Appx00359 at 2:20-25. 10X's own products follow this approach. Appx01306. This is consistent with the testimony from 10X's expert who said it is possible to apply a stimulus to the gel bead without applying it to the oligonucleotide attached to the gel bead. Appx10105-10107 at 414:6-415:8, 416:13-417:9, 418:7-419:2. If the bead and oligonucleotide were one in the same thing, then this would be impossible.[8]

Because the accused stimulus is applied to the oligonucleotide in Bio-Rad's accused systems, Bio-Rad does not infringe the '024 Patent, and no substantial evidence supports a finding otherwise.

III. THE COMMISSION ERRED IN AFFIRMING INFRINGEMENT OF THE '468 PATENT

No substantial evidence supports infringement under the plain language of the claims of the '468 Patent. It was error for the Commission to find otherwise. Appx00051. The claim requires a microfluidic device with two junctions. At the ***first*** junction, two different aqueous fluids are brought together—(1) the sample-

---

[7] Appx00359 at 2:27-31 ("[T]he microcapsule may comprise a bead"). Claim 1 requires it to be a porous gel bead.

[8] 10X's expert tried to walk away from this admission by using a head and hair analogy. That led to the absurd claim that every time one gets a haircut, they are actually getting a "head cut." Appx10119-10120 at 467:9-16, 471:23-472:11 ("Q. So when I cut my hair, I'm cutting my hair, not my head; correct? A. I think it would probably be ***more accurately specified as a head cut***, but you are cutting part of your head.").

containing fluid, and (2) the fluid with oligonucleotide barcodes and reagents. The claim requires that these two liquids form "an aqueous mixture" that travels along the microfluidic channel where it encounters a ***second*** junction. At this second junction, oil intersects with the "aqueous mixture" and encapsulates the "aqueous mixture" into a droplet.

A. The Evidence At Trial Showed Bio-Rad's Aqueous Solutions Must Not Be Mixed Until Droplets Form

Bio-Rad's microfluidic devices include two channel junctions. At the first junction, two channels intersect: one contains sample-containing fluid (e.g., biological cells); and the other contains Bio-Rad's reagent fluid, including beads, oligonucleotide barcodes, and other chemicals that will interact with the sample. Appx03229; Appx02165-02166. These liquid components are loaded into separate wells on the chip (Appx02165-02166); they must be kept separate because each component includes reagents that will start to react if mixed together. Appx10104 at 409:12-18. For instance, the oligonucleotide-bead solution contains a detergent that will lyse the cells in the sample solution if the two aqueous solutions are mixed. Appx03230.

The Bio-Rad device is carefully designed to keep these fluids separate as they travel a very short distance under pressure to a second channel junction on the device where the oil phase is introduced. Appx03229. As the two aqueous fluids encounter the oil phase, they are "pinched off" by the oil to create the droplets. *Id.* The design

parameters of the device, including channel size, liquid pressure, result in uniformly-sized droplets each containing a single cell. Appx03228-03230.

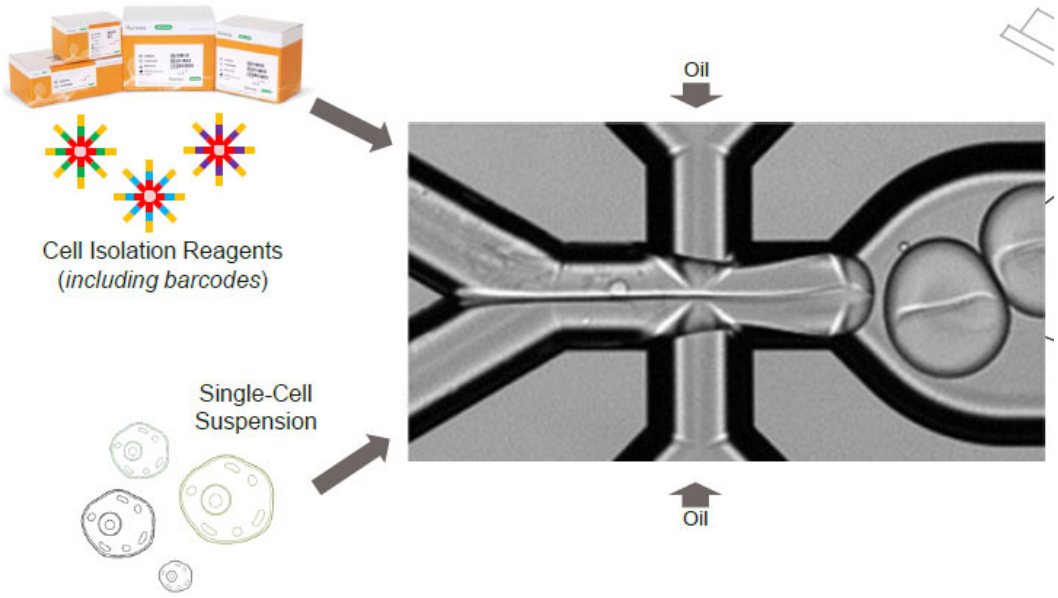
10X's own expert agreed the two aqueous solutions—the sample solution with cells and the barcode solution with detergent—are kept separate and not mixed until they are encapsulated in a droplet. Appx10104 at 408:6-12.

B. The Commission Erred In Affirming The Existence Of An "Aqueous Mixture" In Bio-Rad's Accused Systems

Nonetheless, the ALJ found infringement with respect to an "aqueous mixture" at the first junction. The Commission adopted the ALJ's reasoning on this issue. Appx00051. This was error.

To support infringement, the ID pointed to evidence that the Bio-Rad systems "combine" two aqueous liquids at the first junction. Appx00201 (citing evidence "that these two solutions are combined"); Appx00203 (citing 10X's expert that "the two solutions 'come together'"). But the fact that the two liquids "come together" is not in dispute. What the claim requires, ***in addition*** to the two liquids coming together, is that they form an "aqueous mixture." Appx00406 at 33:55-34:9. Claim limitations cannot be ignored, *PAR Pharm., Inc. v. TWI Pharm., Inc.*, 773 F.3d 1186, 1196 (Fed. Cir. 2014) (vacating because the "district court's analysis . . . ignores the claim limitations at issue"), and there is simply no proof of any "aqueous mixture" occurring at the first junction in Bio-Rad's devices.

This is not a distinction without a difference. Liquids do not instantaneously mix together, especially when they are moving under pressure in microscopic-sized channels. One can combine liquids, and yet they do not mix, as can be visibly seen in the following image depicting Bio-Rad's accused system:



Appx02207. This image shows the two aqueous solutions are separate as they flow from the first junction to the second junction (a line appearing between them), and do not mix until after droplet formation, where the line begins to buckle and the solutions mix for the first time. It was 10X's burden to prove an "aqueous mixture" forms before the second junction, and it did not meet that burden. *See, e.g.*, *Creative Compounds, LLC v. Starmark Labs.*, 651 F.3d 1303, 1314 (Fed. Cir. 2011) (patentee bears the burden).

Not only did 10X fail to offer proof of a mixture of liquids before droplets form, but the evidence demonstrated otherwise. This is because Bio-Rad includes

reagents that react immediately with the contents of the other solution. If they mixed, the entire system would not work because sample would not be partitioned before chemical reactions began. It would be, in the words of 10X's expert, a ***"big mess."*** Appx10104 at 408:10-13; *see also id.* at 409:12-18 ("To be really clear, if I took the one and the other and mixed them without forming a droplet, yes, it would be a big mess.").

10X's expert Dr. Butte further testified that "even a small amount of cell lysis before encapsulation [in a droplet]" would cause the contents of the cell to "be released and mixed with other contents," which would defeat the purpose of single cell studies." Appx10117 at 459:5-23.

10X's expert is exactly right: the cells cannot be lysed before droplets form because the point of the system is to isolate and partition single cells in droplets so individualized chemical reactions can occur. The '468 Patent claim, however, is directed to a different system where aqueous fluids may mix before the droplets are formed. Bio-Rad's system is quite distinct from that type of system. The finding of infringement on this patent must be reversed.

IV. THE COMMISSION ERRED IN FINDING A VIOLATION OF THE '530 PATENT

The Commission erred in finding a violation with respect to the '530 Patent because (1) there is no substantial evidence that either 10X's domestic industry

products[9] or Bio-Rad's accused products practice the '530 Patent, and (2) the shifting claim constructions demonstrate the claims are indefinite.

A. The Commission's Construction Of The '530 Patent Requires Droplet Generation Before Barcode Detachment And Barcoding

The critical issue is the order of steps of the claimed method: first providing the raw materials of gel beads and cells; then generating at least 1,000 droplets that each contain a single cell and its polynucleotides, and a single gel bead with barcodes attached; ***and then*** detaching a plurality of barcodes from "said at least 1,000 gel beads " in order to barcode polynucleotide molecules from the single cell, in each of the at least 1,000 droplets. Appx00450-00451 at 47:58-49:4.

The ALJ's claim construction order provided that the claim "requires that all of the 'at least 1,000 droplets' be generated before the third step of the claim is performed on ***any*** of 'said at least 1,000 droplets.'" Appx00662 ("Interpreting claim 1 otherwise would—as Staff correctly notes—negate the claim's numerical limitations.").

The ALJ later adjusted this construction, ruling "[t]he claim language merely requires that ***any*** accused step of generating a plurality of barcoded molecules occurs after the at least 1,000 droplets are generated." Appx00690.

The Commission's construction of claim 1 of the '530 Patent requires that

[9] To obtain relief from the ITC, 10X was obligated to prove that it practices its own patents in the U.S., *i.e.*, the domestic industry requirement. 19 U.S.C. § 1337(a)(2).

these three steps be completed in order. Appx00099 ("step (b) [must] be completed before step (c)," and affirming the ID's construction which "does not permit an assembly-line style process where step (c) is completed on a droplet as soon as it is generated in step (b)"). The Commission construed step (c) to require a showing that barcode detachment and barcoding of DNA occurs in ***at least 1,000 droplets*** after step (b) is completed. *See, e.g.*, Appx00080.

In 10X's domestic industry products, however, as well as Bio-Rad's accused products, the reagents interact immediately as the droplets form, and there is no proof that a plurality of barcodes detach to barcode a plurality of polynucleotides in each of ***at least 1,000 droplets*** after all 1,000 droplets are formed (the completion of step (b)). This is fatal to 10X's allegations.

B. No Substantial Evidence Supports The Commission's Finding That 10X's Domestic Industry Products Or Bio-Rad's Accused Products Practice The '530 Patent

10X had the burden of proof with respect to domestic industry and infringement; it failed in both cases.

1. 10X Did Not Meet Its Domestic Industry Burden

(a) The Commission Correctly Found That ID Was Not Supported

In 10X's system, 10X includes a chemical that dissolves the gel bead "immediately" upon contact that releases the barcodes, as soon as the droplets are formed. In other words, the barcodes detach from the beads before the at least 1,000

droplets are collected off the chip and moved to the thermocycler. *See, e.g.*, Appx01557; Appx01791 at 5:48-6:08; Appx10103-10104 at 406:17-407:1; Appx03259; Appx01681 (cited at Appx00256); Appx01365-01366 (cited at Appx00082). This is shown in an explanatory video 10X supplies to its customers. Appx01791. The accompanying narrative states: "dissolved beads will appear in the emulsions ***almost immediately*** after GEM [droplet] formation." *Id.* at 5:48-6:08.

Despite this trial record, the ID found 10X's products practiced the '530 Patent. Appx00254-00257. This was error, and on review, the Commission found the ID's conclusion was unsupported, noting the evidence the ID relied on "does not address whether barcodes are released in the domestic industry products after at least 1,000 droplets have been generated as required by step (b) of the asserted claims." Appx00082. The Commission's conclusion should have ended the matter. It erred in finding otherwise.

(b) The Commission Erred In *Sua Sponte* Reviewing The Record To Find Evidence Of Domestic Industry

Despite the ID's lack of supporting evidence, the Commission took upon itself to determine domestic industry, stating: "Here, where the evidence cited by the ID does not support the ID's finding, such *sua sponte* review is appropriate." Appx00082-00083. This led to error—reliance on technically unsound theories and documents that were never tested at trial and for which Bio-Rad had no opportunity to respond.

The Commission's improper approach was two-fold. First, it attempted to calculate the number of minutes it took to generate droplets before the droplets were collected and moved to a thermocycler for PCR amplification. Appx00083-00084. Second—and this is the critical error—it attempted to calculate how long the gel beads survived before dissolving, not based on any technical evidence or expert opinion, but a single slide from an investment presentation unmoored to any actual commercial product. Appx00084 (citing Appx01429; Appx01503). This does not and cannot meet 10X's burden of proof.

(c) The Commission's Reliance On An Investment Slide Unconnected To The Products Was Error

The Commission identified the key issue to be the timing of when the gel beads dissolve in 10X's system and thus detach the barcodes. *See* Appx00084. The Commission relied on a slide from a 10X investor presentation to answer this question. *See* Appx00084-00087 (citing Appx01429; Appx01503). Relying on this slide, the Commission found 10X satisfied its burden. Appx00084-00087. This was error.

First, there is no evidence this slide represents how 10X's commercially-available domestic industry products actually function. The slide is from an ***investor presentation from 2013***. Appx01394; *see also* Appx01503 (same slide reproduced in a 2014 investor presentation). 10X's GemCode products were ***first sold in 2015***, and its Chromium products were ***first sold in 2016***. Appx00036; Appx01237;

Appx02099. ***None*** of 10X's witnesses, either fact or expert, ever testified or provided any explanation of the contents of this slide. There is simply no evidence in the record connecting this slide to any of 10X's domestic industry products.

Second, the investor presentation slide does not provide any technical details regarding barcode detachment. For example, 10X's commercial products use specific chemicals to dissolve the gel beads. *See, e.g.*, Appx01305. The investor presentation does not mention these chemicals anywhere in the entire deck. Appx01394-01476; Appx01477-01542. The slide also does not provide any information regarding the mechanism for dissolution of the gel beads, what reagents were used at what concentrations, whether the size and composition of the gel beads reflect 10X's commercial gel beads, whether the gel beads shown had barcodes attached, how many barcodes are being released at any point in time (if any barcodes were even attached), where the images shown in the slide came from, or how the time measurements were conducted. Post-trial speculation cannot substitute for technical evidence.

Third, Bio-Rad was never afforded any opportunity to respond to this slide with respect to domestic industry. Rather, the only time 10X cited to this slide is in its ***very last submission to the Commission***—its reply submission to the Commission's request for additional briefing. Appx00083 (citing only 10X's reply). The parties conducted an entire claim construction, pretrial, trial, and post-trial

process before the ALJ; yet 10X never once argued or presented this evidence in support of domestic industry. This document is untested, unexamined, and unsupported by any fact or expert testimony—it is not substantial evidence. If any party had disclosed such a document on the eve of trial, it would have been rightly excluded as untimely.[10]

Fourth, the Commission found that the information in the investor presentation slide was "consistent with the testimony of Dr. Schnall-Levin, who testified on cross-examination that the gel bead does not disappear instantaneously after droplet formation." Appx00087-00088. But Dr. Schnall-Levin, a current 10X employee, was not definitive and simply provided a single-sentence conjecture: "No, I don't think so." Appx00087-00088 (citing Appx10057 at 224:18-23). There was no further explanation, and no foundation that this employee knew how 10X's system works regarding gel bead dissolution, and certainly no testimony from any expert on this point.

Lastly, the technical evidence of record shows that in 10X's ***commercial*** (not pre-launch) products, 10X's gel beads dissolve and release barcodes ***immediately*** after being encapsulated in a droplet (which 10X calls a "GEM"). *See, e.g.*, Appx01557 (Chromium Single Cell User Guide: "***Immediately*** following generation

[10] The documents are in the record only because a different portion was cited by a witness to support secondary considerations for validity. Appx02022-02023.

of a GEM, the Single Cell 5′ Gel Bead is dissolved and any co-partitioned cell is lysed."); Appx01791 at 5:48-6:08; Appx10103-10104 at 406:17-407:1; Appx03259. The evidence of record thus shows that barcode detachment occurs immediately in 10X's commercial products. There is simply no evidence to support the Commission's finding that the "at least 1,000 good droplets" that are generated in the last minute in 10X's commercial products "still contain gel beads with attached barcodes." Appx00083-00084.

The Commission's finding with respect to domestic industry for the '530 Patent should therefore be reversed.

2. No Substantial Evidence Supports The Commission's Finding That Bio-Rad Infringes The '530 Patent

The Commission's finding that Bio-Rad's accused products infringe the '530 Patent should be similarly reversed for a failure of proof. There is no dispute that barcoding begins as soon as droplets form in Bio-Rad's accused products. Appx00080 ("[B]oth cleavage and barcoding by reverse transcription happen immediately after droplet formation and at room temperature."). Because the claim requires the steps be performed in order, 10X alleged that at some point after the process begins, at least 1,000 droplets have some barcoding occurring in them on the thermocycler. *See* Appx00076. There is no substantial evidence to support that theory—merely speculation. 10X's expert conducted no testing. And there are no documents showing this happens in Bio-Rad's accused products.

Ignoring this, the Commission relied on three pieces of evidence in finding infringement. *See* Appx00079 (citing Appx02631-02632; Appx03231; Appx02290). But none of this evidence provides ***any quantitative information*** at all regarding how much barcode detachment and barcoding occurs in ***any*** of the at least 1,000 droplets after they are collected and placed on the thermocycler. *See* Appx02631-02632; Appx03231; Appx02290. The only conclusion that can be drawn from this evidence is that ***some amount*** of barcode detachment and barcoding may occur on the thermocycler. But none of this evidence provides any support for the Commission's finding that a plurality of barcodes detach and barcode a plurality of polynucleotides in each of at least 1,000 droplets placed on the thermocycler in Bio-Rad's accused products, as required by the Commission's construction of step (c).

Indeed, the technical evidence shows the opposite: barcoding takes place "almost immediately." Appx03254; Appx03255; Appx03445-03447; Appx03447 at Q/A 18 ("All of these reactions happen extremely rapidly in each droplet as it forms.").

Moreover, the undisputed evidence is that the ddSEQ v1 generates only 1,200 droplets with a single cell and gel bead. *See* Appx00095 (Bio-Rad's ddSEQ v1 chip "generates approximately 1,200 droplets"); Appx00237. Thus, since barcoding begins immediately, there is no evidence that Bio-Rad's products barcode a plurality

of polynucleotides in each of ***at least 1,000 droplets*** on the thermocycler. The Commission affirmed the ID's finding that "***the bulk*** of cleavage and barcoding occur on the thermal cycler" (Appx00080), but such a finding is insufficient to demonstrate cleavage and barcoding occurs in ***at least 1,000 droplets*** on the thermocycler—especially where the ddSEQ generates only 1,200 droplets and ***immediately*** begins cleaving barcodes and barcoding DNA after droplets form. Even if the requisite barcoding occurred in 80% of the Bio-Rad droplets on the thermocycler (much more than the "bulk," and for which there is no evidence), that would still not amount to the necessary barcoding in ***at least 1,000 droplets*** (0.8 x 1,200 = 960 droplets). The Commission's decision should be reversed.

C. The Commission's Construction Rendered The '530 Patent Claims Indefinite

A patent is invalid for indefiniteness "if its claims, read in light of the specification delineating the patent, and the prosecution history, fail to inform, with reasonable certainty, those skilled in the art about the scope of the invention." *Nautilus, Inc. v. Biosig Instruments, Inc.*, 572 U.S. 898, 901 (2014).

Here, the Commission's final construction following conflicting prior constructions render the claims of the '530 Patent indefinite—does step (b) have to be complete before step (c) begins per the original claim construction? Or can step (c) begin in some droplets? And if step (c) can begin right away, how many droplets must form before step (c) can begin? These are not abstract questions. The answer

leads to different infringement results.

The Commission's final construction differed from prior constructions, demonstrating indefiniteness.

The ALJ's *Markman* order repeatedly found "claim 1 requires that the step of generating 'at least 1,000 droplets' be ***completed before*** the third step of forming a 'plurality of barcoded polynucleotide molecules' is performed in ***any*** of the droplets." Appx00664; *see also* Appx00660-00665; Appx00073. This was the basis upon which Bio-Rad prepared for trial.

The ALJ's Order No. 35 changed this requirement and instead found that the '530 Patent does ***not*** "require that all 1,000 droplets form before any barcoding begins . . . . The claim language merely requires that ***any*** accused step of generating a plurality of barcoded molecules occurs after the at least 1,000 droplets are generated." Appx00690; *see also* Appx00074.

The ID then changed the construction again with respect to the order of the steps. In some statements, the ID seemed to go back to its *Markman* order: "Although the step of generating droplets with a cell and gel bead must be ***completed before the start of the third step***, the third step does not require at least 1,000 droplets having a cell and a gel bead . . . . [T]he claim language is still satisfied so long as at least 1,000 of such droplets had been generated ***before the start of the third step***." Appx00238; *see also* Appx00232.

The ID also changed the requirement regarding how much barcode detachment and barcoding is required in step (c), finding that "barcoded polynucleotides" must be generated in at least "1,000 droplets during the incubation step." Appx00240; *see also* Appx00243 (finding there would be no infringement if "all the barcoded molecules have been cleaved from the gel bead and/or the reverse transcriptase has finished forming barcoded cDNA in a sufficient number of droplets so that these processes occur in ***less than 1,000 droplets during incubation***."); *cf.* Appx00690; Appx00243-00244.

Finally, the Commission once again changed the construction of the '530 Patent, and even applied two different constructions when analyzing Bio-Rad's accused products compared with 10X's domestic industry products. For Bio-Rad's products, the Commission affirmed infringement based on a finding that ***any amount of barcoding*** occurred on the thermocycler, and that "***the bulk***" of barcoding occurred on the thermocycler. *See, e.g.*, Appx00079-00080. But with respect to 10X's domestic industry products, the Commission required proof that barcode detachment was occurring in ***at least 1,000 droplets*** in step (c). *See, e.g.*, Appx00080; Appx00084; Appx00087.

Given the shifting and contradictory claim constructions, there was no way for Bio-Rad and the public to know how to design around these claims. *Nautilus*, 572 U.S. at 909 (a patent "must be precise enough to afford clear notice of what is

claimed, thereby apprising the public of what is still open to them.") (internal quotation marks and citations omitted). Otherwise, "there would be a zone of uncertainty" (*id.*), which is the consequence of the Commission's decision here. The claims of the '530 Patent are indefinite.

CONCLUSION

For the foregoing reasons, Bio-Rad requests that the Court reverse the Commission's findings below.

DATED: August 17, 2020 Respectfully submitted,

/s/ *Brian C. Cannon*

Brian C. Cannon
Kevin P.B. Johnson
QUINN EMANUEL URQUHART & SULLIVAN, LLP
555 Twin Dolphin Drive, 5th Floor
Redwood Shores, California 94065-2139
Telephone: (650) 801-5000
Facsimile: (650) 801-5100
briancannon@quinnemanuel.com
kevinjohnson@quinnemanuel.com

David Bilsker
Nathan Sun
Andrew Naravage
QUINN EMANUEL URQUHART & SULLIVAN, LLP
50 California Street, 22nd Floor
San Francisco, California 94111-4788
Telephone: (415) 875-6600
Facsimile: (415) 875-6700
davidbilsker@quinnemanuel.com
nathansun@quinnemanuel.com
andrewnaravage@quinnemanuel.com

S. Alex Lasher
QUINN EMANUEL URQUHART & SULLIVAN, LLP
1300 I Street NW, Suite 900
Washington, DC 20005-3314
Telephone: (202) 538-8104
Facsimile: (202) 538-8100
alexlasher@quinnemanuel.com

Sean T. Gloth
QUINN EMANUEL URQUHART & SULLIVAN, LLP
51 Madison Avenue, 22nd Floor
New York, New York 10010
Telephone: (212) 849-7468
Facsimile: (212) 849-7100
seangloth@quinnemanuel.com

Counsel for Appellant Bio-Rad Laboratories, Inc.

PROOF OF SERVICE

The undersigned hereby certifies that on August 17, 2020, I caused the foregoing PRINCIPAL BRIEF FOR APPELLANT BIO-RAD (confidential and nonconfidential versions) to be electronically filed with the Clerk of the Court for the United States Court of Appeals for the Federal Circuit by using the appellate CM/ECF system. Service will be accomplished via electronic mail. Additionally, the confidential version will be served by email on this same date.

Via CM/ECF and email

Benjamin Richards
Wayne W. Herrington
Office of the General Counsel
U.S. International Trade Commission
500 E Street, S.W.
Washington, D.C. 20436
benjamin.richards@usitc.gov
wayne.herrington@usitc.gov

Via CM/ECF and email

Matthew D. Powers
Paul T. Ehrlich
Stefani C. Smith
Robert L. Gerrity
Jennifer K. Robinson
Utsav Gupta
Daniel M. Radke
TENSEGRITY LAW GROUP, LLP
555 Twin Dolphin Drive, Suite 650
Redwood Shores, CA 94065
Telephone: (650) 802-6000
Facsimile: (650) 802-6001
10x_tlg_service@tensegritylawgroup.com

Azra M. Hadzimehmedovic
Aaron M. Nathan
Samantha A. Jameson
TENSEGRITY LAW GROUP, LLP
8260 Greensboro Drive, Suite 260
McLean, VA 22102
Telephone: (650) 802-6000
Facsimile: (650) 802-6001
10x_tlg_service@tensegritylawgroup.com

Counsel for Appellee International Trade Commission

Counsel for Appellant 10X Genomics, Inc.

Upon acceptance by the Court of the e-filed document, six confidential paper copies will be filed with the Court within the time provided in the Court's rules.

/s/ *Brian C. Cannon*
Brian C. Cannon

CERTIFICATE OF COMPLIANCE
WITH TYPE-VOLUME LIMITATIONS

The foregoing filing complies with the relevant type-volume limitation of the Federal Rules of Appellate Procedure and Federal Circuit Rules because it has been prepared using a proportionally-spaced typeface and includes 13,878 words.

Dated: August 17, 2020

/s/ *Brian C. Cannon*
Brian C. Cannon

CERTIFICATE OF CONFIDENTIAL MATERIAL

The foregoing document contains 2 unique words (including numbers) marked confidential. This number does not exceed the maximum of 15 words permitted by Fed. Cir. R. 25.1(d)(1)(A).

Dated: August 17, 2020

/s/ *Brian C. Cannon*
Brian C. Cannon

2020-1785

United States Court of Appeals for the Federal Circuit

BIO-RAD LABORATORIES, INC.,
Appellant

v.

INTERNATIONAL TRADE COMMISSION,
Appellee

10X GENOMICS, INC.,
Intervenor

Appeals from the United States International Trade Commission in Investigation No. 337-TA-1100.

NONCONFIDENTIAL ADDENDUM TO THE PRINCIPAL BRIEF FOR APPELLANT BIO-RAD

Brian C. Cannon
Kevin P.B. Johnson
QUINN EMANUEL URQUHART & SULLIVAN, LLP
555 Twin Dolphin Drive, 5th Floor
Redwood Shores, California 94065
Telephone: (650) 801-5000
briancannon@quinnemanuel.com
kevinjohnson@quinnemanuel.com

Counsel for Appellant Bio-Rad Laboratories, Inc.

David Bilsker
Nathan Sun
Andrew Naravage
QUINN EMANUEL URQUHART & SULLIVAN, LLP
50 California Street, 22nd Floor
San Francisco, California 94111
Telephone: (415) 875-6600
davidbilsker@quinnemanuel.com
nathansun@quinnemanuel.com
andrewnaravage@quinnemanuel.com

S. Alex Lasher
QUINN EMANUEL URQUHART & SULLIVAN, LLP
1300 I Street NW, Suite 900
Washington, DC 20005
Telephone: (202) 538-8104
alexlasher@quinnemanuel.com

Sean T. Gloth
QUINN EMANUEL URQUHART & SULLIVAN, LLP
51 Madison Avenue, 22nd Floor
New York, New York 10010
Telephone: (212) 849-7468
seangloth@quinnemanuel.com

Counsel for Appellant Bio-Rad Laboratories, Inc.

TABLE OF CONTENTS

This Nonconfidential Addendum contains information that has been designated confidential business information subject to the protective order in the underlying Investigation and that has been removed from this version of the appendix pursuant to Federal Circuit Rule 25.1. The omitted confidential material concerns certain non-public product specifications, business plans, and business practices relating to the parties and their products. The applicable page numbers are: Appx00035, Appx00054-00065, Appx00068, Appx00071, Appx00076-00082, Appx00087-00089, Appx00103-00105, Appx00107, Appx00112, Appx00113, Appx00123-00125, Appx00127, Appx00129, Appx00130, Appx00133, Appx00135, Appx00144, Appx00159-00171, Appx00173, Appx00192-00199, Appx00202-00205, Appx00207, Appx00213, Appx00215, Appx00216, Appx00218, Appx00219, Appx00223, Appx00224, Appx00227-00229,

Appx00232-00237, Appx00239-00249, Appx00251-00253, Appx00255, Appx00256, Appx00259, Appx00261-00276, Appx00283-00290, Appx00292, and Appx00293.

UNITED STATES INTERNATIONAL TRADE COMMISSION

Washington, D.C.

In the Matter of	
CERTAIN MICROFLUIDIC SYSTEMS AND COMPONENTS THEREOF AND PRODUCTS CONTAINING SAME	**Inv. No. 337-TA-1100**

ORDER NO. 1: PROTECTIVE ORDER

(February 15, 2018)

WHEREAS, documents and information may be sought, produced or exhibited by and among the parties to the above captioned proceeding, which materials relate to trade secrets or other confidential research, development or commercial information, as such terms are used in the Commission's Rules, 19 C.F.R. § 210.5;

IT IS HEREBY ORDERED THAT:

1. Confidential business information is information which concerns or relates to the trade secrets, processes, operations, style of work, or apparatus, or to the production, sales, shipments, purchases, transfers, identification of customers, inventories, amount or source of any income, profits, losses, or expenditures of any person, firm, partnership, corporation, or other organization, or other information of commercial value, the disclosure of which is likely to have the effect of either (i) impairing the Commission's ability to obtain such information as is necessary to perform its statutory functions; or (ii) causing substantial harm to the competitive position of the person, firm, partnership, corporation, or other organization from which the information was obtained, unless the Commission is required by law to disclose such

information. The term "confidential business information" includes "proprietary information" within the meaning of section 777(b) of the Tariff Act of 1930 (19 U.S.C. § 1677f(b)).

2(a). Any information submitted, in pre-hearing discovery or in a pleading, motion, or response to a motion either voluntarily or pursuant to order, in this investigation, which is asserted by a supplier to contain or constitute confidential business information shall be so designated by such supplier in writing, or orally at a deposition, conference or hearing, and shall be segregated from other information being submitted. Documents shall be clearly and prominently marked on their face with the legend: "CONFIDENTIAL BUSINESS INFORMATION SUBJECT TO PROTECTIVE ORDER" or a comparable notice. Such information, whether submitted in writing or in oral testimony, shall be treated in accordance with the terms of this protective order.

(b). The Administrative Law Judge or the Commission may determine that information alleged to be confidential is not confidential, or that its disclosure is necessary for the proper disposition of the proceeding, before, during or after the close of a hearing herein. If such a determination is made by the Administrative Law Judge or the Commission, opportunity shall be provided to the supplier of such information to argue its confidentiality prior to the time of such ruling.

3. In the absence of written permission from the supplier or an order by the Commission or the Administrative Law Judge, any confidential documents or business information submitted in accordance with the provisions of paragraph 2 above shall not be disclosed to any person other than: (i) outside counsel for parties to this investigation, including necessary secretarial and support personnel assisting such counsel; (ii) qualified persons taking testimony involving such documents or information and necessary stenographic and clerical

personnel thereof; (iii) technical experts and their staff who are employed for the purposes of this litigation (unless they are otherwise employed by, consultants to, or otherwise affiliated with a non-governmental party, or are employees of any domestic or foreign manufacturer, wholesaler, retailer, or distributor of the products, devices or component parts that are the subject of this investigation); (iv) the Commission, the Administrative Law Judge, the Commission staff, and personnel of any governmental agency as authorized by the Commission; (v) the Commission, its employees, and contract personnel who are acting in the capacity of Commission employees, for developing or maintaining the records of this investigation or related proceedings for which this information is submitted, or in internal audits and investigations relating to the programs and operations of the Commission pursuant to 5 U.S.C. Appendix 3; and (vi) by U.S. government employees and contract personnel, solely for cybersecurity purposes.[1]

4. Confidential business information submitted in accordance with the provisions of paragraph 2 above shall not be made available to any person designated in paragraph 3(i)[2] and (iii) unless he or she shall have first read this order and shall have agreed, by letter filed with the Secretary of this Commission: (i) to be bound by the terms thereof; (ii) not to reveal such confidential business information to anyone other than another person designated in paragraph 3; (iii) to utilize such confidential business information solely for purposes of this investigation; and (iv) including the following acknowledgment:

> I, the undersigned, acknowledge that information submitted in response to this request for information and throughout this investigation or other proceeding may be disclosed to and used:
>
> (i) by the Commission, its employees and Offices, and contract personnel (a) for developing or maintaining the records of this or a related proceeding, or (b) in internal investigations, audits, reviews, and evaluations relating to the programs,

[1] *See* Commission Administrative Order 16-01 (Nov. 7, 2015).
[2] Necessary secretarial and support personnel assisting counsel need not sign onto the protective order themselves because they are covered by counsel's signing onto the protective order.

personnel, and operations of the Commission including under 5 U.S.C. Appendix 3; or

(ii) by U.S. government employees and contract personnel, solely for cybersecurity purposes. I understand that all contract personnel will sign appropriate nondisclosure agreements.

5. If the Commission or the Administrative Law Judge orders, or if the supplier and all parties to the investigation agree, that access to, or dissemination of information submitted as confidential business information shall be made to persons not included in paragraph 3 above, such matter shall only be accessible to, or disseminated to, such persons based upon the conditions pertaining to, and obligations arising from this order, and such persons shall be considered subject to it, unless the Commission or the Administrative Law Judge finds that the information is not confidential business information as defined in paragraph 1 hereof.

6(a). Any confidential business information submitted to the Commission or the Administrative Law Judge in connection with a motion or other proceeding within the purview of this investigation shall be submitted under seal pursuant to paragraph 2 above. Any portion of a transcript in connection with this investigation containing any confidential business information submitted pursuant to paragraph 2 above shall be bound separately and filed under seal. When any confidential business information submitted in accordance with paragraph 2 above is included in an authorized transcript of a deposition or exhibits thereto, arrangements shall be made with the court reporter taking the deposition to bind such confidential portions and separately label them "CONFIDENTIAL BUSINESS INFORMATION SUBJECT TO PROTECTIVE ORDER." Before a court reporter or translator receives any such information, he or she shall have first read this order and shall have agreed in writing to be bound by the terms thereof. Alternatively, he or she shall sign the agreement included as Attachment A hereto.

Copies of each such signed agreement shall be provided to the supplier of such confidential business information and the Secretary of the Commission.

(b) Suppliers of confidential business information are strongly encouraged to encrypt nonpublic documents that are electronically transmitted to the Commission to protect your sensitive information from unauthorized disclosure. The USITC secure drop-box system and the Electronic Document Information System (EDIS) use Federal Information Processing Standards (FIPS) 140-2 cryptographic algorithms to encrypt data in transit. Submitting your nonpublic documents by a means that does not use these encryption algorithms (such as by email) may subject your firm's nonpublic information to unauthorized disclosure during transmission. If you choose a non-encrypted method of electronic transmission, the Commission warns you that the risk of such possible unauthorized disclosure is assumed by you and not by the Commission.

7. The restrictions upon, and obligations accruing to, persons who become subject to this order shall not apply to any information submitted in accordance with paragraph 2 above to which the person asserting the confidential status thereof agrees in writing, or the Commission or the Administrative Law Judge rules, after an opportunity for hearing, was publicly known at the time it was supplied to the receiving party or has since become publicly known through no fault of the receiving party.

8. The Commission, the Administrative Law Judge, and the Commission investigative attorney acknowledge that any document or information submitted as confidential business information pursuant to paragraph 2 above is to be treated as such within the meaning of 5 U.S.C. § 552(b)(4) and 18 U.S.C. § 1905, subject to a contrary ruling, after hearing, by the Commission or its Freedom of Information Act Officer, or the Administrative Law Judge. When such information is made part of a pleading or is offered into the evidentiary record, the data set

forth in 19 C.F.R. § 201.6 must be provided except during the time that the proceeding is pending before the Administrative Law Judge. During that time, the party offering the confidential business information must, upon request, provide a statement as to the claimed basis for its confidentiality.

9. Unless a designation of confidentiality has been withdrawn, or a determination has been made by the Commission or the Administrative Law Judge that information designated as confidential, is no longer confidential, the Commission, the Administrative Law Judge, and the Commission investigative attorney shall take all necessary and proper steps to preserve the confidentiality of, and to protect each supplier's rights with respect to, any confidential business information designated by the supplier in accordance with paragraph 2 above, including, without limitation: (a) notifying the supplier promptly of (i) any inquiry or request by anyone for the substance of or access to such confidential business information, other than those authorized pursuant to this order, under the Freedom of Information Act, as amended (5 U.S.C. § 552) and (ii) any proposal to redesignate or make public any such confidential business information; and (b) providing the supplier at least seven days after receipt of such inquiry or request within which to take action before the Commission, its Freedom of Information Act Officer, or the Administrative Law Judge, or otherwise to preserve the confidentiality of and to protect its rights in, and to, such confidential business information.

10. If while an investigation is before the Administrative Law Judge, a party to this order who is to be a recipient of any business information designated as confidential and submitted in accordance with paragraph 2 disagrees with respect to such a designation, in full or in part, it shall notify the supplier in writing, and they will thereupon confer as to the status of the subject information proffered within the context of this order. If prior to, or at the time of such a

conference, the supplier withdraws its designation of such information as being subject to this order, but nonetheless submits such information for purposes of the investigation, such supplier shall express the withdrawal, in writing, and serve such withdrawal upon all parties and the Administrative Law Judge. If the recipient and supplier are unable to concur upon the status of the subject information submitted as confidential business information within ten days from the date of notification of such disagreement, any party to this order may raise the issue of the designation of such a status to the Administrative Law Judge who will rule upon the matter. The Administrative Law Judge may *sua sponte* question the designation of the confidential status of any information and, after opportunity for hearing, may remove the confidentiality designation.

11. No less than 10 days (or any other period of time designated by the Administrative Law Judge) prior to the initial disclosure to a proposed expert of any confidential information submitted in accordance with paragraph 2, the party proposing to use such expert shall submit in writing the name of such proposed expert and his or her educational and detailed employment history to the supplier. If the supplier objects to the disclosure of such confidential business information to such proposed expert as inconsistent with the language or intent of this order or on other grounds, it shall notify the recipient in writing of its objection and the grounds therefore prior to the initial disclosure. If the dispute is not resolved on an informal basis within ten days of receipt of such notice of objections, the supplier shall submit immediately each objection to the Administrative Law Judge for a ruling by filing a motion for a protective order pursuant to Commission Rule 210.34. If the investigation is before the Commission the matter shall be submitted to the Commission for resolution. The submission of such confidential business information to such proposed expert shall be withheld pending the ruling of the Commission or the Administrative Law Judge. The terms of this paragraph shall be inapplicable

to experts within the Commission or to experts from other governmental agencies who are consulted with or used by the Commission.

12. If confidential business information submitted in accordance with paragraph 2 is disclosed to any person other than in the manner authorized by this protective order, the party responsible for the disclosure must immediately bring all pertinent facts relating to such disclosure to the attention of the supplier and the Administrative Law Judge and, without prejudice to other rights and remedies of the supplier, make every effort to prevent further disclosure by it or by the person who was the recipient of such information.

13. Nothing in this order shall abridge the right of any person to seek judicial review or to pursue other appropriate judicial action with respect to any ruling made by the Commission, its Freedom of Information Act Officer, or the Administrative Law Judge concerning the issue of the status of confidential business information.

14. Upon final termination of this investigation, each recipient of confidential business information that is subject to this order shall assemble and return to the supplier all items containing such information submitted in accordance with paragraph 2 above, including all copies of such matter which may have been made. Alternatively, the parties subject to this order may, with the written consent of the supplier, destroy all items containing confidential business information and certify to the supplier (or his counsel) that such destruction has taken place. This paragraph shall not apply to the Commission, including its investigative attorney, and the Administrative Law Judge, which shall retain such material pursuant to statutory requirements and for other recordkeeping purposes, but may destroy such material (including electronic media containing such information) in its possession which it regards as surplusage.

Notwithstanding the above paragraph, confidential business information may be transmitted to a district court pursuant to Commission Rule 210.5(c).

15. If any confidential business information which is supplied in accordance with paragraph 2 above is supplied by a nonparty to this investigation, such a nonparty shall be considered a "supplier" as that term is used in the context of this order.

16. Each nonparty supplier shall be provided a copy of this order by the party seeking information from said supplier.

17. The Secretary shall serve a copy of this order upon all parties.

SO ORDERED.

Dee Lord
Dee Lord
Administrative Law Judge

Attachment A

NONDISCLOSURE AGREEMENT FOR REPORTER/STENOGRAPHER/TRANSLATOR

I, _____ , do solemnly swear or affirm that I will not divulge any information communicated to me in any confidential portion of the investigation or hearing in the matter of *Certain Microfluidic Systems and Components Thereof and Products Containing Same*, Investigation No. 337-TA-1100, except as permitted in the protective order issued in this case. I will not directly or indirectly use, or allow the use of such information for any purpose other than that directly associated with my official duties in this case.

Further, I will not by direct action, discussion, recommendation, or suggestion to any person reveal the nature or content of any information communicated during any confidential portion of the investigation or hearing in this case.

I also affirm that I do not hold any position or official relationship with any of the participants in said investigation.

I am aware that the unauthorized use or conveyance of information as specified above is a violation of the Federal Criminal Code and punishable by a fine of up to $10,000, imprisonment of up to ten (10) years, or both.

Signed

Dated

Firm or affiliation

UNITED STATES INTERNATIONAL TRADE COMMISSION
Washington, D.C.

In the Matter of **CERTAIN MICROFLUIDIC SYSTEMS AND COMPONENTS THEREOF AND PRODUCTS CONTAINING SAME**	**Investigation No. 337-TA-1100**

NOTICE OF THE COMMISSION'S FINAL DETERMINATION FINDING A VIOLATION OF SECTION 337; ISSUANCE OF A LIMITED EXCLUSION ORDER AND CEASE AND DESIST ORDER; AND TERMINATION OF THE INVESTIGATION.

AGENCY: U.S. International Trade Commission.

ACTION: Notice.

SUMMARY: Notice is hereby given that the U.S. International Trade Commission has determined that there is a violation of 19 U.S.C. 1337, as amended ("section 337"), in the above-captioned investigation. The Commission has further determined to issue a limited exclusion order and cease and desist order and to set a bond rate on the entered value of covered products imported during the period of Presidential review.

FOR FURTHER INFORMATION CONTACT: Benjamin S. Richards, Esq., Office of the General Counsel, U.S. International Trade Commission, 500 E Street SW, Washington, DC 20436, telephone (202) 708-5453. Copies of non-confidential documents filed in connection with this investigation are or will be available for inspection during official business hours (8:45 a.m. to 5:15 p.m.) in the Office of the Secretary, U.S. International Trade Commission, 500 E Street SW, Washington, DC 20436, telephone (202) 205-2000. General information concerning the Commission may also be obtained by accessing its Internet server at *https://www.usitc.gov*. The public record for this investigation may be viewed on the Commission's electronic docket (EDIS) at *https://edis.usitc.gov*. Hearing-impaired persons are advised that information on this matter can be obtained by contacting the Commission's TDD terminal on (202) 205-1810.

SUPPLEMENTARY INFORMATION: On February 21, 2018, the Commission instituted this investigation based on a complaint filed by 10X Genomics, Inc. of Pleasanton, CA. 83 Fed. Reg. 7491 (Feb. 21, 2018). The complaint alleges violations of section 337 of the Tariff Act of 1930, as amended, 19 U.S.C. 1337, in the importation into the United States, the sale for importation, or the sale within the United States after importation of certain microfluidic systems and components thereof and products containing same by reason of infringement of one or more claims of U.S. Patent Nos. 9,644,204 ("the '204 patent"); 9,689,024 ("the '024 patent"); 9,695,468 ("the '468 patent"); and 9,856,530 ("the '530 patent"). *Id.* The Commission's

notice of investigation named as the sole respondent Bio-Rad Laboratories, Inc. of Hercules, CA. *Id.* The Office of Unfair Import Investigations ("OUII") is participating in this investigation. *Id.*

On July 12, 2019, the administrative law judge ("ALJ") issued the final initial determination ("ID"). The ID found a violation of section 337 by virtue of Bio-Rad's indirect infringement of the '024, the '468, and the '530 patents. The ID found that 10X had not established a violation with respect to the '204 patent. The ID also found that Bio-Rad failed to establish invalidity of any of the asserted claims of any patent. The ID further found that the domestic industry requirement was satisfied for each of the asserted patents. Finally, the ID found that Bio-Rad had not carried its burden with respect to various additional affirmative defenses, including improper inventorship and ownership.

On July 25, 2019, the ALJ issued her recommended determination on remedy and bonding. The ALJ recommended, upon a finding of violation, that the Commission issue a limited exclusion order, issue a cease and desist order, and impose a bond in the amount of twenty-five percent of the entered value of any covered products imported during the period of Presidential review.

On July 29, 2019, 10X, Bio-Rad, and OUII submitted petitions seeking review of the ID. On August 6, 2019, 10X, Bio-Rad, and OUII submitted responses to the others' petitions. On August 26, 2019, 10X and Bio-Rad submitted comments on the public interest pursuant to Commission Rule 210.50(a)(4).

On October 17, 2019, the Commission issued a notice indicating its determination to review the ID with respect to (1) all findings related to a violation based on the '024 patent; (2) all findings related to a violation based on the '468 patent; (3) noninfringement of the '204 patent; (4) all findings related to a violation based on the '530 patent; (5) Bio-Rad's inventorship and ownership defenses; and (6) a typographical error on page 91. The same notice also requested briefing from the parties on certain of those issues, and on remedy, bonding, and the public interest. The notice also included an extension of the target date to December 19, 2019.

The parties filed their initial responses to the Commission's questions on October 31, 2019, and their replies on November 7, 2019.

Upon review of the parties' submissions, the ID, RD, and evidence of record, the Commission has determined that Bio-Rad violated section 337 by reason of infringement of asserted claims 1, 5, 17, 19, and 22 of the '024 patent, claims 1, 6, 7, 9, and 21 of the '468 patent, and claims 1, 4, 11, 14, 19, 26, and 28 of the '530 patent. The Commission found no violation with respect to the '240 patent. The Commission has further determined to issue a limited exclusion order prohibiting further importation of Bio-Rad's infringing microfluidic systems and a cease and desist order against Bio-Rad. The Commission will set a bond of twenty-five percent of entered value on Bio-Rad's infringing microfluidic systems imported during the period of Presidential review.

The authority for the Commission's determination is contained in section 337 of the Tariff Act of 1930, as amended (19 U.S.C. 1337), and in part 210 of the Commission's Rules of Practice and Procedure (19 CFR 210).

By order of the Commission.

Lisa R. Barton
Secretary to the Commission

Issued: February 12, 2020

CERTAIN MICROFLUIDIC SYSTEMS AND COMPONENTS THEREOF AND PRODUCTS CONTAINING SAME

Inv. No. 337-TA-1100

PUBLIC CERTIFICATE OF SERVICE

I, Lisa R. Barton, hereby certify that the attached **NOTICE** has been served by hand upon the Commission Investigative Attorney, **Monica Bhattacharyya, Esq.,** and the following parties as indicated, on **February 12, 2020.**

Lisa R. Barton, Secretary
U.S. International Trade Commission
500 E Street, SW, Room 112
Washington, DC 20436

On Behalf of Complainants 10X Genomics, Inc.:

Paul T. Ehrlich
TENSEGRITY LAW GROUP LLP
555 Twin Dolphin Dr., Suite 650
Redwood Shores, CA 94061

☐ Via Hand Delivery
☒ Via Express Delivery
☐ Via First Class Mail
☐ Other:_____

On Behalf of Respondents Bio-Rad Laboratories, Inc.:

S. Alex Lasher
QUINN EMANUEL URQUHART & SULLIVAN, LLP
1300 I Street NW, Suite 900
Washington, DC 20005

☐ Via Hand Delivery
☒ Via Express Delivery
☐ Via First Class Mail
☐ Other:_____

UNITED STATES INTERNATIONAL TRADE COMMISSION
Washington, D.C.

In the Matter of **CERTAIN MICROFLUIDIC SYSTEMS AND COMPONENTS THEREOF AND PRODUCTS CONTAINING SAME**	**Investigation No. 337-TA-1100**

LIMITED EXCLUSION ORDER

The Commission has determined that there is a violation of section 337 of the Tariff Act of 1930, as amended (19 U.S.C. 1337), in the unlawful importation, sale for importation, and/or sale within the United States after importation by Bio-Rad Laboratories, Inc. of Hercules, California ("Bio-Rad" or "Respondent") of certain microfluidic systems and components thereof and products containing same that infringe one or more of claims 1, 5, 17, 19, and 22 of U.S. Patent No. 9,689,024 ("the '024 patent"); claims 1, 6, 7, 9, and 21 of U.S. Patent No. 9,695,468 ("the '468 patent"); and claims 1, 4, 11, 14, 19, 26, and 28 of U.S. Patent No. 9,856,530 ("the '530 patent").

Having reviewed the record of this investigation, including the written submissions of the parties, the Commission has made its determination on the issues of remedy, the public interest, and bonding. The Commission has determined that the appropriate form of relief includes a limited exclusion order prohibiting the unlicensed entry of covered microfluidic systems and components thereof and products containing same manufactured by or on behalf of, or imported by or on behalf of, Respondent or any of its affiliated companies, parents, subsidiaries, or other

related business entities, or its successors or assigns. This Exclusion Order does not apply to microfluidic consumables[1] imported into the United States for use by researchers who are using such consumables in the United States as of the date of issuance of this Order, and who have a documented need to continue receiving the consumables for a specific current ongoing research project for which that need cannot be met by any alternative product.

The Commission has also determined that the public interest factors enumerated in 19 U.S.C. § 1337(d)(1) do not preclude the issuance of this limited exclusion order. Finally, the Commission has determined that the bond during the Presidential review period shall be in the amount of twenty-five (25) percent of the entered value for all covered products.

Accordingly, the Commission hereby **ORDERS** that:

1. Microfluidic systems and components thereof and products containing same that infringe one or more of claims 1, 5, 17, 19, and 22 of the '024 patent; claims 1, 6, 7, 9, and 21 of the '468 patent; and claims 1, 4, 11, 14, 19, 26, and 28 of the '530 patent, and that are manufactured by or on behalf of, or imported by or on behalf of, Respondent or any of its affiliated companies, parents, subsidiaries, or other related business entities, or their successors or assigns ("covered products"), are excluded from entry for consumption into the United States, entry for consumption from a foreign trade zone, or withdrawal from a warehouse for consumption, for the remaining terms of the patents, except under license of the patent owner or as provided by law.

[1] "Consumable" means any otherwise covered Bio-Rad part or material that is purchased for use with Bio-Rad's droplet generation instruments and which is consumed during the use of those instruments. For example, Bio-Rad's microfluidic chips are consumables.

2. The provisions of this Order shall not apply to covered consumables imported into the United States for use by researchers who are using such consumables in the United States as of the date of issuance of this Order, and who have a documented need[2] to continue receiving the consumables for a specific current ongoing research project for which that need cannot be met by any alternative product. The provisions of this Order shall also not apply to service or repair articles imported for use in servicing or repairing microfluidic systems that were imported as of the date of this Order and are under a warranty that existed as of the date of this Order, if such servicing or repairing is provided for in terms of the warranty.
3. Notwithstanding paragraph 1 of this Order, the covered products are entitled to entry into the United States for consumption, entry for consumption from a foreign-trade zone, or withdrawal from a warehouse for consumption under bond in the amount of twenty-five (25) percent of the entered value of such articles pursuant to subsection (j) of Section 337 (19 U.S.C. § 1337(j)) and the Presidential Memorandum for the United States Trade Representative of July 21, 2005 (70 Fed. Reg. 43,251), from the day after this Order is received by the United States Trade Representative until such time as the United States Trade Representative notifies the Commission that this Order is approved or disapproved but, in any event, not later than sixty (60) days after the date of receipt of this Order. All entries of covered products made pursuant to this paragraph are to be reported to U.S. Customs and Border Protection ("CBP"), in

[2] This "documented need" is to be satisfied by the questionnaire attached to this Order, as discussed at pages 84–86 of the Commission Opinion issued in this investigation on the date of this Order. Bio-Rad is not required to maintain the individual researchers' records supporting the questionnaire. Commission Opinion, at 85–86.

advance of the date of the entry, pursuant to procedures CBP establishes.

4. At the discretion of CBP and pursuant to procedures that it establishes, persons seeking to import microfluidic systems and components thereof and products containing same that are potentially subject to this Order may be required to certify that they are familiar with the terms of this Order, that they have made appropriate inquiry, and thereupon state that, to the best of their knowledge and belief, the products being imported are not excluded from entry under paragraph 1 of this Order. At its discretion, CBP may require persons who have provided the certification described in this paragraph to furnish such records or analyses as are necessary to substantiate the certification.
5. In accordance with 19 U.S.C. § 1337(1), the provisions of this Order shall not apply to covered products that are imported by and for the use of the United States, or imported for, and to be used for, the United States with the authorization or consent of the Government.
6. The Commission may modify this Order in accordance with the procedures described in Rule 210.76 of the Commission's Rules of Practice and Procedure (19 C.F.R. § 210.76).
7. The Secretary shall serve copies of this Order upon each party of record in this

Investigation and upon CBP.

8. Notice of this Order shall be published in the *Federal Register*.

By order of the Commission.

Lisa R. Barton
Secretary to the Commission

Issued: February 12, 2020

ATTACHMENT

Name: _____

Institution: _____

If you were conducting research using Bio-Rad's ddSEQ consumables as of February 12, 2020, in the United States and you need to continue to receive the ddSEQ consumables for that research, answer the following questions:

1. What is the subject matter of your research that uses Bio-Rad's ddSEQ system and consumables?

2. On what date (mm/dd/yyyy) did your research using these Bio-Rad systems begin?

3. What is the expected completion date (mm/dd/yyyy) of your research that uses these Bio-Rad systems?

4. What other competing products did you consider for your research, and why did you reject these products?

I certify that all information provided as part of this questionnaire is accurate and complete to the best of my knowledge. I am aware that U.S. law (including, but not limited to, 18 U.S.C. § 1001) imposes criminal sanctions on individuals who knowingly and willfully make material false statements to the U.S. Government.

I acknowledge that I am to maintain records supporting the above declarations and am not to provide those supporting records to Bio-Rad. If the facts change concerning my research, which began on or before February 12, 2020, I understand that I am to provide an updated questionnaire response to Bio-Rad.

Date: _____ Signature: _____

Additional Bio-Rad comments [to be completed by Bio-Rad]:

I certify that all information provided as part of this questionnaire is accurate and complete to the best of my knowledge. I am aware that U.S. law (including, but not limited to, 18 U.S.C. 1001) imposes criminal sanctions on individuals who knowingly and willfully make material false statements to the U.S. Government.

Date: _____ Signature: _____

CERTAIN MICROFLUIDIC SYSTEMS AND COMPONENTS THEREOF AND PRODUCTS CONTAINING SAME

Inv. No. 337-TA-1100

PUBLIC CERTIFICATE OF SERVICE

I, Lisa R. Barton, hereby certify that the attached **COMMISSION ORDER** has been served by hand upon the Commission Investigative Attorney, **Monica Bhattacharyya, Esq.,** and the following parties as indicated, on **February 12, 2020.**

Lisa R. Barton, Secretary
U.S. International Trade Commission
500 E Street, SW, Room 112
Washington, DC 20436

On Behalf of Complainants 10X Genomics, Inc.:

Paul T. Ehrlich
TENSEGRITY LAW GROUP LLP
555 Twin Dolphin Dr., Suite 650
Redwood Shores, CA 94061

☐ Via Hand Delivery
☒ Via Express Delivery
☐ Via First Class Mail
☐ Other:_____

On Behalf of Respondents Bio-Rad Laboratories, Inc.:

S. Alex Lasher
QUINN EMANUEL URQUHART & SULLIVAN, LLP
1300 I Street NW, Suite 900
Washington, DC 20005

☐ Via Hand Delivery
☒ Via Express Delivery
☐ Via First Class Mail
☐ Other:_____

UNITED STATES INTERNATIONAL TRADE COMMISSION
Washington, D.C.

In the Matter of **CERTAIN MICROFLUIDIC SYSTEMS AND COMPONENTS THEREOF AND PRODUCTS CONTAINING SAME**	**Investigation No. 337-TA-1100**

CEASE AND DESIST ORDER

IT IS HEREBY ORDERED THAT Bio-Rad Laboratories, Inc. of Hercules, California cease and desist from conducting any of the following activities in the United States: importing, selling, marketing, advertising, distributing, transferring (except for exportation), and soliciting U.S. agents or distributors for, or aiding and abetting other entities in the importation, sale for importation, sale after importation, transfer (except for exportation), or distribution of microfluidic systems and components thereof and products containing same covered by one or more of claims 1, 5, 17, 19, and 22 of U.S. Patent No. 9,689,024 ("the '024 patent"); claims 1, 6, 7, 9, and 21 of U.S. Patent No. 9,695,468 ("the '468 patent"); and claims 1, 4, 11, 14, 19, 26, and 28 of U.S. Patent No. 9,856,530 ("the '530 patent") in violation of Section 337 of the Tariff Act of 1930, as amended (19 U.S.C. § 1337).

I.
Definitions

As used in this order:

(A) "Commission" shall mean the United States International Trade Commission.

(B) "Complainant" shall mean 10X Genomics, Inc. of Pleasanton, California.

(C) "Respondent" shall mean Bio-Rad Laboratories, Inc., of Hercules, California.

(D) "Person" shall mean an individual, or any non-governmental partnership, firm, association, corporation, or other legal or business entity other than Respondent or its majority owned or controlled subsidiaries, successors, or assigns.

(E) "United States" shall mean the fifty States, the District of Columbia, and Puerto Rico.

(F) The terms "import" and "importation" refer to importation for entry for consumption under the Customs laws of the United States.

(G) The term "covered products" shall mean microfluidic systems and components thereof and products containing same that infringe one or more of claims 1, 5, 17, 19, and 22 of the '024 patent; claims 1, 6, 7, 9, and 21 of the '468 patent; and claims 1, 4, 11, 14, 19, 26, and 28 of the '530 patent.[1] "Covered products" shall not include articles for which a provision of law or license avoids liability for infringement of all asserted claims of the Asserted Patents.

(H) The term "consumable" means any otherwise covered Bio-Rad part or material that is purchased for use with Bio-Rad's droplet generation instruments and which is consumed during the use of those instruments. For example, Bio-Rad's microfluidic chips are consumables.

II.
Applicability

The provisions of this Cease and Desist Order shall apply to Respondent and to any of its principals, stockholders, officers, directors, employees, agents, distributors, controlled (whether

[1] For purposes of this Order, "covered products" includes products for which associated conduct and/or inventory is permitted based on a documented need.

by stock ownership or otherwise) and majority-owned business entities, successors, and assigns, and to each of them, insofar as they are engaging in conduct prohibited by section III, *infra*, for, with, or otherwise on behalf of, Respondent.

III.
Conduct Prohibited

The following conduct of Respondent in the United States is prohibited by this Order. For the remaining term of one of the '024, '468, and '530 patents, Respondent shall not:

(A) import, sell for importation into the United States, or sell after importation covered products;

(B) market, distribute, offer to sell, or otherwise transfer (except for exportation) in the United States imported covered products;

(C) advertise imported covered products;

(D) solicit U.S. agents or distributors for imported covered products; or

(E) aid or abet other entities in the importation, sale for importation, sale after importation, transfer, or distribution of imported covered products.

IV.
Conduct Permitted

Notwithstanding any other provision of this Order, specific conduct otherwise prohibited by the terms of this order shall be permitted if: (1) in a written instrument, the owner of the '024, '468, and '530 patents licenses or authorizes such specific conduct; (2) the conduct is limited to service or repair articles imported for use in servicing or repairing microfluidic systems that were imported as of the date of this Order and are under a warranty that existed as of the date of this Order, if such servicing or repairing is provided for in terms of the warranty; or (3) such specific conduct is related to the importation or sale of covered products by or for the United States. This Order does not prohibit the importation or sale of covered microfluidic consumables for use by

researchers who are using such consumables in the United States as of the date of the issuance of this Order, and who have a documented need[2] to continue receiving the consumables for a specific current ongoing research project for which that need cannot be met by any alternative product.

V.
Reporting

For purposes of this requirement, the reporting periods shall commence on the first day of each calendar month and shall end on the last day of each calendar month. The first report required under this section shall cover the period from the date of issuance of this order through the last day of that calendar month.

Within five (5) days of the last day of each month's reporting period, Respondent shall report to the Commission: (a) the quantity in units and the value in dollars of covered products that it has (i) imported and/or (ii) sold in the United States after importation during the reporting period, (b) the quantity in units and the value in dollars of covered products imported and/or sold for use in each research project for which there is a documented need pursuant to Section IV and the identity of each such purchaser, (c) questionnaires[3] from each such purchaser supporting the documented need pursuant to Section IV, and (d) the quantity in units and value in dollars of reported covered products that remain in inventory in the United States at the end of the reporting period.

When filing written submissions, Respondent must file the original document

---

[2] This "documented need" is to be satisfied by the questionnaire attached to this Order, as discussed at pages 84–86 of the Commission Opinion issued in this investigation on the date of this Order. Bio-Rad is not required to maintain the individual researchers' records supporting the questionnaire. Commission Opinion, at 85–86.

[3] *See* Footnote 2.

electronically on or before the deadlines stated above and submit eight (8) true paper copies to the Office of the Secretary by noon the next day pursuant to section 210.4(f) of the Commission's Rules of Practice and Procedure (19 C.F.R. § 210.4(f)). Submissions should refer to the investigation number ("Inv. No. 337-TA-1100") in a prominent place on the cover pages and/or the first page. (*See* Handbook for Electronic Filing Procedures, *https://www.usitc.gov/documents/handbook_on_filing_procedures.pdf*). Persons with questions regarding filing should contact the Office of the Secretary (202-205-2000). If Respondent desires to submit a document to the Commission in confidence, it must file the original and a public version of the original with the Office of the Secretary and must serve a copy of the confidential version on Complainant's counsel.[4]

Any failure to make the required report or the filing of any false or inaccurate report shall constitute a violation of this Order, and the submission of a false or inaccurate report may be referred to the U.S. Department of Justice as a possible criminal violation of 18 U.S.C. § 1001.

VI.
Recordkeeping and Inspection

(A) For the purpose of securing compliance with this Order, Respondent shall retain any and all records relating to the sale, offer for sale, marketing, or distribution in the United States of covered products, made and received in the usual and ordinary course of business (including documents related to the documented need to continue receiving consumables for a specific current ongoing research project provided in Section IV), whether in detail or in summary form, for a period of three (3) years

[4] Complainant must file a letter with the Secretary identifying the attorney to receive reports associated with this order. The designated attorney must be on the protective order entered in the investigation.

from the close of the fiscal year to which they pertain.

(B) For the purposes of determining or securing compliance with this Order and for no other purpose, subject to any privilege recognized by the federal courts of the United States, and upon reasonable written notice by the Commission or its staff, duly authorized representatives of the Commission shall be permitted access and the right to inspect and copy, in Respondent's principal office during office hours, and in the presence of counsel or other representatives if Respondent so chooses, all books, ledgers, accounts, correspondence, memoranda, and other records and documents, in detail and in summary form, that must be retained under subparagraph VI(A) of this Order.

VII.
Service of Cease and Desist Order

Respondent is ordered and directed to:

(A) Serve, within fifteen days after the effective date of this Order, a copy of this Order upon each of its respective officers, directors, managing agents, agents, and employees who have any responsibility for the importation, marketing, distribution, sale of imported covered products in the United States;

(B) Serve, within fifteen days after the succession of any persons referred to in subparagraph VII(A) of this order, a copy of the order upon each successor; and

(C) Maintain such records as will show the name, title, and address of each person upon whom the order has been served, as described in subparagraphs VII(A) and VII(B) of this order, together with the date on which service was made.

The obligations set forth in subparagraphs VII(B) and VII(C) shall remain in effect until the expiration dates of the '024, '468, and '530 patents.

VIII.
Confidentiality

Any request for confidential treatment of information obtained by the Commission pursuant to section V–VI of this order should be made in accordance with section 201.6 of the Commission's Rules of Practice and Procedure (19 C.F.R. § 201.6). For all reports for which confidential treatment is sought, Respondent must provide a public version of such report with confidential information redacted.

IX.
Enforcement

Violation of this order may result in any of the actions specified in section 210.75 of the Commission's Rules of Practice and Procedure (19 C.F.R. § 210.75), including an action for civil penalties under section 337(f) of the Tariff Act of 1930 (19 U.S.C. § 1337(f)), as well as any other action that the Commission deems appropriate. In determining whether Respondent is in violation of this order, the Commission may infer facts adverse to Respondent if it fails to provide adequate or timely information.

X.
Modification

The Commission may amend this order on its own motion or in accordance with the procedure described in section 210.76 of the Commission's Rules of Practice and Procedure (19 C.F.R. § 210.76).

XI.
Bonding

The conduct prohibited by Section III of this Order may be continued during the sixty-day period in which this Order is under review by the United States Trade Representative, as delegated by the President (70 Fed. Reg. 43,251 (Jul. 21, 2005)) subject to the Respondent's posting of a bond in the amount of twenty-five (25) percent of the entered value of the covered

products. This bond provision does not apply to conduct that is otherwise permitted by section IV of this order. Covered products imported on or after the date of issuance of this order are subject to the entry bond set forth in the exclusion order issued by the Commission, and are not subject to this bond provision.

The bond is to be posted in accordance with the procedures established by the Commission for the posting of bonds by complainants in connection with the issuance of temporary exclusion orders. *See* 19 C.F.R. § 210.68. The bond and any accompanying documentation are to be provided to and approved by the Commission prior to the commencement of conduct that is otherwise prohibited by section III of this Order. Upon the Secretary's acceptance of the bond, (a) the Secretary will serve an acceptance letter on all parties, and (b) Respondent must serve a copy of the bond and any accompanying documentation on Complainant's counsel.[5]

The bond is to be forfeited in the event that the United States Trade Representative approves this Order (or does not disapprove it within the review period), unless the U.S. Court of Appeals for the Federal Circuit, in a final judgment, reverses any Commission final determination and order as to Respondent on appeal, or unless Respondent exports or destroys the products subject to this bond and provides certification to that effect that is satisfactory to the Commission.

[5] *See* Footnote 4.

The bond is to be released in the event the United States Trade Representative disapproves this order and no subsequent order is issued by the Commission and approved (or not disapproved) by the United States Trade Representative, upon service on Respondent of an order issued by the Commission based upon application therefore made by Respondent to the Commission.

By order of the Commission.

Lisa R. Barton
Secretary to the Commission

Issued: February 12, 2020

ATTACHMENT

Name: _____

Institution: _____

If you were conducting research using Bio-Rad's ddSEQ consumables as of February 12, 2020, in the United States and you need to continue to receive the ddSEQ consumables for that research, answer the following questions:

1. What is the subject matter of your research that uses Bio-Rad's ddSEQ system and consumables?

2. On what date (mm/dd/yyyy) did your research using these Bio-Rad systems begin?

3. What is the expected completion date (mm/dd/yyyy) of your research that uses these Bio-Rad systems?

4. What other competing products did you consider for your research, and why did you reject these products?

I certify that all information provided as part of this questionnaire is accurate and complete to the best of my knowledge. I am aware that U.S. law (including, but not limited to, 18 U.S.C. 1001) imposes criminal sanctions on individuals who knowingly and willfully make material false statements to the U.S. Government.

I acknowledge that I am to maintain records supporting the above declarations and am not to provide those supporting records to Bio-Rad. If the facts change concerning my research, which began on or before February 12, 2020, I understand that I am to provide an updated questionnaire response to Bio-Rad.

Date: _____ Signature: _____

Additional Bio-Rad comments [to be completed by Bio-Rad]:

I certify that all information provided as part of this questionnaire is accurate and complete to the best of my knowledge. I am aware that U.S. law (including, but not limited to, 18 U.S.C. 1001) imposes criminal sanctions on individuals who knowingly and willfully make material false statements to the U.S. Government.

Date: _____ Signature: _____

CERTAIN MICROFLUIDIC SYSTEMS AND COMPONENTS THEREOF AND PRODUCTS CONTAINING SAME

Inv. No. 337-TA-1100

PUBLIC CERTIFICATE OF SERVICE

I, Lisa R. Barton, hereby certify that the attached **COMMISSION ORDER** has been served by hand upon the Commission Investigative Attorney, **Monica Bhattacharyya, Esq.,** and the following parties as indicated, on **February 12, 2020.**

Lisa R. Barton, Secretary
U.S. International Trade Commission
500 E Street, SW, Room 112
Washington, DC 20436

On Behalf of Complainants 10X Genomics, Inc.:

Paul T. Ehrlich
TENSEGRITY LAW GROUP LLP
555 Twin Dolphin Dr., Suite 650
Redwood Shores, CA 94061

☐ Via Hand Delivery
☒ Via Express Delivery
☐ Via First Class Mail
☐ Other:_____

On Behalf of Respondents Bio-Rad Laboratories, Inc.:

S. Alex Lasher
QUINN EMANUEL URQUHART & SULLIVAN, LLP
1300 I Street NW, Suite 900
Washington, DC 20005

☐ Via Hand Delivery
☒ Via Express Delivery
☐ Via First Class Mail
☐ Other:_____

PUBLIC VERSION

UNITED STATES INTERNATIONAL TRADE COMMISSION
Washington, D.C.

In the Matter of

CERTAIN MICROFLUIDIC SYSTEMS AND COMPONENTS THEREOF AND PRODUCTS CONTAINING SAME

Investigation No. 337-TA-1100

COMMISSION OPINION

PUBLIC VERSION

I. INTRODUCTION

On October 17, 2019, the Commission determined to review portions of the Administrative Law Judge's ("ALJ") final initial determination, which issued on July 12, 2019. 84 Fed. Reg. 56835 (Oct. 23, 2019). On review, the Commission has determined that respondent Bio-Rad Laboratories, Inc. of Hercules, CA ("Bio-Rad" or "Respondent") violated section 337 of the Tariff Act of 1930, 19 U.S.C. § 1337, as amended ("Section 337"), by way of infringement of certain claims of U.S. Patent No. 9,689,024 ("the '024 patent"), U.S. Patent No. 9,695,468 ("the '468 patent"), and U.S. Patent No. 9,856,530 ("the '530 patent"). The Commission has also determined that there is no violation with respect to U.S. Patent No. 9,644,204 ("the '204 patent"). The Commission has determined to issue a limited exclusion order ("LEO") and a cease and desist order ("CDO") against Bio-Rad. The Commission has further determined that during the period of Presidential review, a bond in the amount of twenty-five (25) percent of entered value shall be applied to Bio-Rad's covered products.

II. BACKGROUND

A. Procedural History

On February 21, 2018, the Commission instituted this investigation based on a complaint filed by 10X Genomics, Inc. of Pleasanton, California ("10X" or "Complainant"). 83 Fed. Reg. 7491 (Feb. 21, 2018). The complaint, as supplemented, alleges violations of Section 337, in the importation into the United States, the sale for importation, or the sale within the United States after importation of certain microfluidic systems and components thereof and products containing same by reason of infringement of one or more claims of the '204 patent; the '024 patent; the '468 patent; and the '530 patent. *Id.* The Commission's notice of investigation named Bio-Rad as the sole respondent. *Id.* The Office of Unfair Import Investigations ("OUII") participated in this investigation. *Id.*

The ALJ granted 10X's unopposed motion for summary determination that it has satisfied the economic prong of the domestic industry requirement. Order No. 19 at 5 (Oct. 5, 2018), *unreviewed*, Notice (Nov. 6, 2018). The ALJ also terminated the investigation with respect to several patent claims. Order No. 26 at 2 (Nov. 30, 2018), *unreviewed*, Notice (Dec. 20, 2018); Order No. 27 at 2 (Dec. 10, 2018), *unreviewed*, Notice (Dec. 21, 2018).

From March 25 to 29, 2019, an evidentiary hearing was held in this investigation. At the hearing, 10X asserted the following claims against Bio-Rad:

Patent	Asserted Claims
'024 Patent	Claims 1, 5, 17, 19, 22
'204 Patent	Claims 27, 29, 31, 33
'468 Patent	Claims 1, 6, 7, 9, 21
'530 Patent	Claims 1, 4, 11, 14, 19, 26, 28

See ID at 16–17, 58, 70, 89; *see also* 10X Posthearing Br. at 4.

On July 12, 2019, the ALJ issued her final initial determination ("ID") on violation. The ID found that Bio-Rad imported into the United States, sold for importation, or sold within the United States after importation "the accused microfluidic systems and components thereof and products containing same." ID at 154. The ID found that Bio-Rad indirectly infringed all of the remaining asserted claims of the '024, '468, and '530 patents, but that 10X had not established that Bio-Rad infringed any asserted claims of the '204 patent. *Id.* The ID found that Bio-Rad failed to establish invalidity of any of the asserted claims of any patent. *Id.* The ID found that the domestic industry requirement was satisfied for each of the asserted patents. *Id.* at 154–55. Finally, the ID found that Bio-Rad had not carried its burden with respect to various additional affirmative defenses, including improper inventorship and ownership. *Id.* at 155. Thus, the ID concluded that Bio-Rad violated Section 337 with respect to the '024, '468, and '530 patents, but not with respect to the '204 patent. *Id.* at 154.

On July 25, 2019, the ALJ issued her recommended determination on remedy and bonding ("RD"). The RD recommended issuance of a limited exclusion order upon a finding of violation, without a certification provision. RD at 1–2. The RD further recommended issuance of a cease and desist order. *Id.* at 2–3. The RD also recommended imposition of a bond of twenty-five (25) percent of the entered value of any covered products during the Presidential review period. *Id.* at 3–5. On July 29, 2019, 10X, Bio-Rad, and OUII submitted petitions seeking review of the ID.[1] On August 6, 2019, 10X, Bio-Rad, and OUII submitted responses to the others' petitions.[2]

On October 17, 2019, the Commission issued a notice of its determination to review the ID in part. Particularly, the Commission determined to review the ID with respect to:

> (1) all findings related to a violation based on the '024 patent; (2) all findings related to a violation based on the '468 patent; (3) noninfringement of the '204 patent; (4) all findings related to a violation based on the '530 patent; (5) Bio-Rad's inventorship and ownership defenses; and (6) a typographical error on page 91.

84 Fed. Reg. 56835. The Commission also requested briefing on multiple issues. *Id.*

[1] Complainant 10X Genomics, Inc.'s Petition for Review of the Initial Determination (July 29, 2019) ("10X Pet."); Respondent Bio-Rad Laboratories, Inc.'s Petition for Review of the Initial Determination on Violation of Section 337 (July 30, 2019) ("Bio-Rad Pet."); Petition of the Office of Unfair Import Investigations for Review of the Initial Determination on Violation of Section 337 (July 29, 2019) ("OUII Pet.").

[2] Complainant 10X Genomics, Inc.'s Response to Respondent Bio-Rad Laboratories, Inc.'s Petition for Review of the Initial Determination on Violation of Section 337 (Aug. 6, 2019) ("10X Resp. to Bio-Rad Pet."); Complainant 10X Genomics, Inc.'s Response to Petition of the Office of Unfair Import Investigations Petition for Review of the Initial Determination on Violation of Section 337 (Aug. 6, 2019) ("10X Resp. to OUII Pet."); Respondent Bio-Rad Laboratories, Inc.'s Combined Response to 10X's and the Office of Unfair Import Investigations' Petitions for Review of the Initial Determination (Aug. 6, 2019) ("Bio-Rad Resp. to Pets."); The Office of Unfair Import Investigations' Combined Response to Petitions for Review of the Initial Determination on Violation of Section 337 (Aug. 6, 2019) ("OUII Resp. to Pets.").

On October 31, 2019, the parties filed their respective responses to the Commission's questions on review.[3] On November 7, 2019, the parties filed their respective replies.[4]

B. Overview of the Technology

The technology at issue in this investigation relates to methods of preparing deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA") samples for genetic sequencing and analysis. Particularly, the technology seeks to preserve certain information about nucleic acid segments that would otherwise be lost during sequencing, *e.g.*, whether two nucleic acid segments originated from the same source. This is accomplished by tagging nucleic acid segments, prior to sequencing, with oligonucleotide "barcodes."[5] These barcodes allow researchers to later identify nucleic acid segments that originated from a common sample. The barcoding process involves partitioning nucleic acids from a sample into droplets along with single gel beads to which oligonucleotide barcodes are attached. The barcodes are released from the gel beads and combined with the nucleic acids. At that point, the nucleic acids in each droplet bear a unique barcode. Those nucleic acids can then be pooled and sequenced, and it will still be possible to associate nucleic acid segments from a common droplet. The partitioning of nucleic acids and gel beads

[3] Complainant 10X Genomics, Inc.'s Opening Written Submission Regarding the Commission's October 17, 2019 Notice (Oct. 31, 2019) ("10X Resp. to Qs."); Respondent Bio-Rad Laboratories, Inc.'s Opening Submission Responding to the Commission's Notice Dated October 17, 2019 (Oct. 31, 2019) ("Bio-Rad Resp. to Qs."); The Office of Unfair Import Investigations' Responses to the Commission's October 17, 2019 Questions (Oct. 31, 2019) ("OUII Resp. to Qs.").

[4] Complainant 10X Genomics, Inc.'s Reply Written Submission Regarding the Commission's October 17, 2019 Notice (Nov. 7, 2019) ("10X Reply"); Respondent Bio-Rad Laboratories, Inc.'s Combined Reply to 10X's and the Office of Unfair Import Investigations' Response to the Commission Notice Dated October 17, 2019 (Nov. 7, 2019) ("Bio-Rad Reply"); The Office of Unfair Import Investigations' Reply to the Private Parties' Responses to the Commission's October 17, 2019 Questions (Nov. 7, 2019) ("OUII Reply").

[5] A "barcode" is a short DNA sequence of 3–12 DNA bases. *See* Bio-Rad Prehearing Br. at 8.

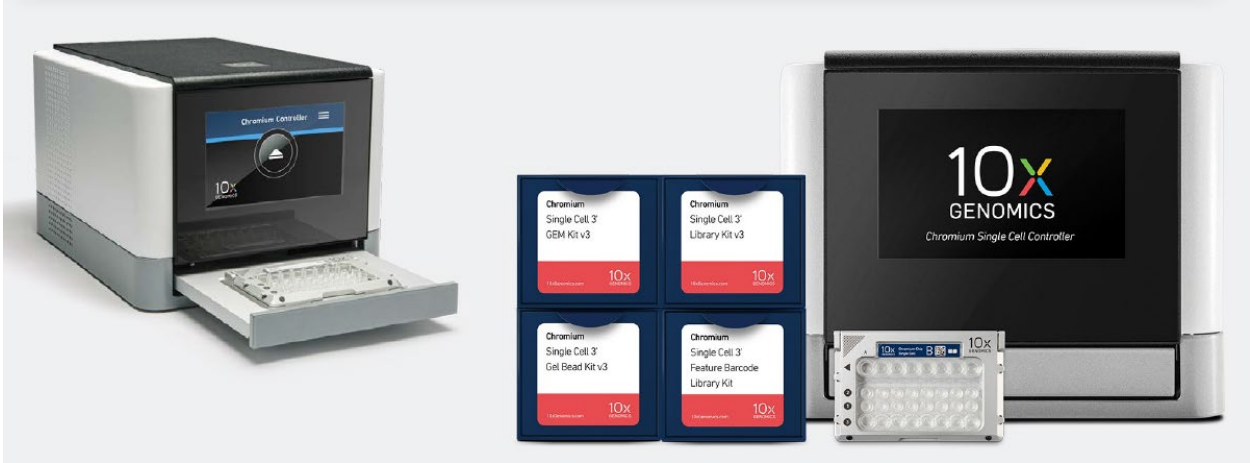
into droplets is accomplished with microfluidic systems that rely on small channels to combine streams of nucleic acids and gel beads into droplets. The asserted claims that remain in this investigation are directed to various aspects of this barcoding process.

C. Products at Issue

The accused products are components and assays of Bio-Rad's ddSEQ system, which includes ddSEQ version 1 and version 2. ID at 3. The ID explained that the ddSEQ v1 products include Bio-Rad's ddSEQ v1 Cartridge, ddSEQ v1 Single-Cell Isolator, ddSEQ Cartridge Holder, and consumables and assays used with and/or as part of Bio-Rad's ddSEQ v1 system, including the SureCell WTA 3' v1 assay. *Id.* (citing CX-0004C (Butte DWS) at Q/A 54; RX-0665C (Metzker RWS) at Q/A 29). The ddSEQ v2 products include [redacted], scATACseq, [redacted]. *Id.* 10X provided the following image of the ddSEQ v1 Single-Cell Isolator and WTA 3' library prep kit products:

See CX-1485C (product launch announcement); CDX-2 at 22 (reproducing CX-1485C).

The domestic industry products are 10X's GemCode™ and Chromium™ product lines. *Id.* at 3. The ID explained that these products were developed by 10X based on its GEM ("Gel bead in Emulsion") architecture, and the first GemCode™ product was sold in 2015. *Id.* (citing CX-0003C at Q/A 47-52). The domestic industry products include both single-cell and linked-read applications, including the Chromium™ Single Cell 3' Solution, Chromium™ Single Cell V(D)J Solution, and GemCode™ Single Cell platform, and the Chromium™ Genome Solution, Chromium™ Exome Solution, Chromium™ de nova Assembly Solution, and GemCode™ Long Read platform. *Id.* 10X provided the following image of its domestic industry products:



See CDX-2 at 80 (reproducing images from 10X's website).

III. THE '024 PATENT

The Commission determined to review all of the ID's findings related to the '024 patent. 84 Fed. Reg. 56835. On review, the Commission has determined to affirm with modified reasoning the ID's finding that Bio-Rad has violated section 337 based on infringement of the '024 patent. Specifically, the Commission finds that Bio-Rad failed to raise the location of amplification as a basis for noninfringement in its petition for review and has therefore abandoned that argument. The Commission further finds that the '024 patent is infringed regardless of whether the claim term "amplification" encompasses reverse transcription, and therefore the Commission need not resolve that dispute as it will not have a material effect on the outcome of this investigation. Concerning invalidity, the Commission affirms the ID's finding that Bio-Rad has not established that any of the asserted claims are invalid under modified reasoning. The Commission adopts the remainder of the ID's findings with respect to the '024 patent to the extent they are not inconsistent with this opinion.

For reference, claim 1 of the '024 patent follows:

1. A method for sample preparation, comprising:

    a) providing a droplet comprising ***a porous gel bead*** and a target nucleic acid analyte, wherein said porous gel bead comprises at least 1,000,000 oligonucleotide molecules comprising barcode sequences, wherein said oligonucleotide molecules are releasably attached to said porous gel bead, wherein said barcode sequences are the same sequence for said oligonucleotide molecules;

    b) applying a stimulus to said porous gel bead to release said oligonucleotide molecules from said porous gel bead into said droplet, wherein upon release from said porous gel bead, a given oligonucleotide molecule from said oligonucleotide molecules attaches to said target nucleic acid analyte; and

    c) subjecting said given oligonucleotide molecule attached to said target nucleic acid analyte to nucleic acid ***amplification*** to yield a barcoded target nucleic acid analyte.

'024 patent at cl. 1 (emphasis added on contested terms).

A. Construction of "Amplification" and the Effect on Infringement

OUII petitioned for review of the ALJ's construction of the term "nucleic acid amplification," which appears in asserted claim 1 of the '024 patent and asserted claim 21 of the '468 patent. *See* OUII Pet. at 18–26. Specifically, OUII asserted that the *Markman* order erred by construing "nucleic acid amplification" such that "creation of a single complementary copy through reverse transcription constitutes 'amplification.'" *Id.* at 20. However, OUII also acknowledged that whether "amplification" should be construed to encompass reverse transcription may be immaterial to the ID's ultimate conclusion that Bio-Rad violated section 337 based on infringement of the '024 patent. *See id.* at 19 ("[T]his issue may not be material since, under the proper construction, the ID's ultimate violation holdings on [the '024 and '468] patents are correct."). OUII elaborated that "10X provided evidence of infringement and the technical prong under both the broader construction adopted by the Court, as well as the narrower

construction supported by OUII," and noted that "the ID appeared to rely on 10X's evidence under both constructions, although the ID focused at times on reverse transcription." *Id.* at 25.

10X disagreed with OUII's assertion that the *Markman* order misconstrued "nucleic acid amplification," 10X Resp. to OUII Pet. at 7–13, but agreed that "under either the ALJ's or Staff's proposed construction of 'amplification,' the findings of violation for the ['']024 and ['']468 Patents are correct and should stand." *Id.* at 13. Particularly, 10X asserted that because no party challenged the ID's infringement findings based on the construction of "amplification," "[OUII]'s challenge to one aspect of the claim construction will have no material effect and any error would be harmless." *Id.*

Bio-Rad did not petition for review of the *Markman* order's construction of "nucleic acid amplification." *See generally* Bio-Rad Pet. Bio-Rad did petition for review of the ID's finding that the asserted claims of the '024 and '468 patents were infringed, but the arguments Bio-Rad advanced in support of that aspect of its petition were based on entirely different limitations in the claims. *See* Bio-Rad Pet. at 6–9, 27–33, 66–73. In its response to OUII's petition, however, Bio-Rad agreed with OUII that the *Markman* order misconstrued "amplification" to encompass reverse transcription. *See* Bio-Rad Resp. to Pets. at 35–38.

Notwithstanding the fact that Bio-Rad did not petition for review of the construction of "nucleic acid amplification," it argued for the first time in its response to OUII's petition that its products do not infringe the '024 patent "under the correct construction of the 'amplification' terms." Bio-Rad Resp. to Pets. at 38. The noninfringement argument Bio-Rad laid out in support of that assertion did not relate to whether "nucleic acid amplification" encompassed reverse transcription, however. *See id.* at 38–40 (no discussion of reverse transcription). Rather, Bio-Rad argued that "claim 1 of the '024 Patent requires that amplification occur in the droplet," and that

the evidence does not show that amplification occurs in a droplet in Bio-Rad's products. *Id.* at 39. In making that argument, Bio-Rad revived a dispute decided in the *Markman* order — whether amplification must occur in a droplet — for which no party sought review. *See* Order No. 22 at 44–45 (rejecting the same Bio-Rad argument and finding that "[t]he requirement that the 'said given oligonucleotide molecule attached to said target nucleic acid analyte' be created in a droplet in the second step does not mean that it has to remain in the droplet for all subsequent steps").

Given the disagreement over the materiality of the construction of "amplification" as set forth in OUII's petition for review, and the apparent disconnect between Bio-Rad's noninfringement argument and the question of whether "amplification" encompasses reverse transcription, the Commission sought briefing from the parties addressing those issues. 84 Fed. Reg. 56836. 10X and OUII both responded that modifying the construction of "amplification" to exclude reverse transcription would have no effect on the ID's infringement findings because the evidence of record shows other multiple types of amplification in the accused products, including polymerase chain reaction ("PCR"), which would meet the definition of "amplification" even if that term did not encompass reverse transcription. 10X Resp. to Qs. at 21–23; OUII Resp. to Qs. at 13. Further, both 10X and OUII responded that whether "amplification" must occur in a droplet and whether "amplification" encompasses reverse transcription are distinct issues and therefore modifying the ID's construction of "amplification" to exclude reverse transcription would not give rise to a noninfringement finding based on the location where amplification occurs. *See* 10X Resp. to Qs. at 23–24; OUII Resp. to Qs. at 14. Accordingly, both 10X and OUII responded that Bio-Rad waived its noninfringement argument based on whether amplification must occur in a droplet. 10X Resp. to Qs. at 26–27; OUII Resp. to Qs. at 14–15.

Bio-Rad responded that "[i]f amplification does not include reverse transcription, than [sic] all but Bio-Rad's scATACseq products do not infringe Claim 1 of the '024 Patent or Claim 21 of the '468 Patent," because reverse transcription is the only amplification reaction that occurs in a droplet in Bio-Rad's products. *See* Bio-Rad Resp. to Qs. at 28. We note that, by taking this position, Bio-Rad expanded its previous noninfringement argument, which was limited to the '024 patent. *See* Bio-Rad Resp. to Pets. at 38. Bio-Rad's briefing in support of its position also included a new argument not previously made in its petition or in response to the other parties' petitions. Particularly, Bio-Rad argued that the "said target nucleic acid analyte" in claim 1 of the '024 patent and claim 21 of the '468 patent must be messenger RNA ("mRNA"), but that in proving infringement 10X relied on complementary DNA ("cDNA") to establish amplification of nucleic acids outside a droplet. *See* Bio-Rad Resp. to Qs. at 29–31.

Concerning waiver, Bio-Rad responded that OUII's petition preserved its noninfringement argument. The crux of Bio-Rad's position in this regard appears to be that by challenging one aspect of the *Markman* order's construction of "amplification" — whether "amplification" encompasses reverse transcription — OUII's petition opened the door for Bio-Rad (or 10X) to challenge other aspects of that construction in its response to OUII's petition. *See id.* at 31–33. Bio-Rad also argued that the ID only relied on reverse transcription as the basis for its infringement finding, and therefore, Bio-Rad was not required to specifically petition for review of whether its products are infringing based on amplification outside the droplet. *See id.* at 33–34. Bio-Rad then submitted that "[i]f the Commission determines that 'amplification' can occur outside of the droplet, the Commission should remand to the ALJ to make specific findings on infringement under that construction." *Id.* at 34. Notably, notwithstanding the Commission's request for "citations to where this [amplification location] issue was raised in Bio-Rad's prehearing brief,

posthearing brief, and petition for review," 84 Fed. Reg. 56836, Bio-Rad provides none in its response to the Commission's waiver question. *See* Bio-Rad Resp. to Qs. at 31–34.

The dispute regarding whether the term "nucleic acid amplification" encompasses reverse transcription is immaterial to any issue in the investigation, and thus the Commission need not resolve that dispute. As the Federal Circuit has explained, "only those terms need be construed that are in controversy, and only to the extent necessary to resolve the controversy." *Vivid Techs., Inc. v. Am. Sci. & Eng'g, Inc.*, 200 F.3d 795, 803 (Fed. Cir. 1999). The Commission need not resolve issues of claim construction that are not material to any issue in this investigation. *See Nidec Motor Corp. v. Zhongshan Broad Ocean Motor Co. Matal*, 868 F.3d 1013, 1017 (Fed. Cir. 2017) ("[W]e need not construe the claim preambles here where the construction is not material to the [obviousness] dispute." (alteration in original) (internal quotation marks omitted)); *EmeraChem Holdings, LLC v. Volkswagen Grp. of Am., Inc.*, 714 F. App'x 995, 997 (Fed. Cir. 2017) (unpublished) (declining to decide claim construction dispute "because the prior art would anticipate the '558 patent claims regardless of which construction we apply.").

The dispute over whether "amplification" should encompass reverse transcription is immaterial because, as noted in the ID, 10X pointed to four different reactions in the accused products to satisfy the "amplification" limitation of claim 1 of the '024 patent. *See* ID at 25–26 ("[Dr. Butte] further explains that barcoded cDNA strands are generated from the oligonucleotide molecules through several different processes, which 10X identifies in its brief as four types of amplification."). One of the processes identified is PCR, which is explicitly listed as an amplification reaction in the '024 patent. *See* '024 patent at 25:25–28 ("[O]ligonucleotide primers containing bar code sequences may be used in amplification reactions (e.g., PCR, qPCR, reverse-transcriptase PCR, digital PCR, etc.) of the DNA template analytes, thereby producing tagged

analytes."). Even Bio-Rad has acknowledged that PCR is a type of amplification reaction. *See* Bio-Rad Initial Claim Construction Br. at 16 (listing evidence where PCR is described as an amplification reaction). While 10X argued in its pre- and post-hearing briefs that PCR in the accused products satisfied the "amplification" limitation in claim 1 of the '024 patent, Bio-Rad did not address whether the PCR relied on by 10X satisfied the "nucleic acid amplification" limitation. *Compare* 10X Prehearing Br. at 33–35; 10X Initial Posthearing Br. at 24–26 *with* Bio-Rad Posthearing Br. at 62–63 (disputing infringement of "amplification" limitation without addressing PCR) *and* Bio-Rad Posthearing Reply at 12 (same). Instead, Bio-Rad limited itself to arguing that "the oligonucleotide molecule containing the barcode that attaches to the target nucleic acid analyte (mRNA) acts as a primer during the reverse transcription reaction," and because "this portion of the oligonucleotide molecule is not amplified in reverse transcription," 10X could not show that the accused products satisfy the "amplification" limitation. Bio-Rad Posthearing Br. at 62–63; *see also* Bio-Rad Posthearing Reply Br. at 12; Bio-Rad Prehearing Br. at 65–68. Bio-Rad never challenged 10X's assertion that the "amplification" limitation is satisfied by PCR. *See generally* 10X Initial Posthearing Br. at 24–26.

Given Bio-Rad's failure to present evidence or argument disputing 10X's evidence and argument that the "amplification" limitation is satisfied by PCR in the accused products, the Commission affirms the ID's finding that the accused products practice the "amplification" limitation. A preponderance of the evidence supports that finding under the broad construction applied in the ID, as well as under a narrow construction that excludes reverse transcription from the definition of "amplification." Accordingly, whether "amplification" should be construed to encompass reverse transcription is not material to any issue in this investigation; the Commission

need not resolve that question and takes no position on it. The Commission affirms the remainder of the ID's infringement findings with respect to the '024 patent.[6]

With respect to the argument regarding whether amplification must occur in a droplet, which Bio-Rad raised as a basis for noninfringement in its response to OUII's petition, Bio-Rad abandoned that argument and waived it by failing to raise it in its petition for review. Commission Rule 210.43(b)(2) states that "[a]ny issue not raised in a petition for review will be deemed to have been abandoned by the petitioning party and may be disregarded by the Commission in reviewing the initial determination . . . and any argument not relied on in a petition for review will be deemed to have been abandoned and may be disregarded by the Commission." 19 C.F.R. § 210.43(b)(2). Further, the ALJ's Ground Rule 8.2 states that "[a]ny contentions not set forth in detail as required herein shall be deemed abandoned or withdrawn, except for contentions of which a party is not aware and could not be aware in the exercise of reasonable diligence at the time of filing the pre-trial brief," while Ground Rule 11.1 states that issues not raised in post-trial briefs "shall be deemed waived." *See* Order No. 2 (Ground Rules). During the *Markman* process, the ALJ resolved three distinct disputes with respect to the meaning of "amplification" in the asserted patents. *See* Order No. 22 at 31–45. Whether "amplification" encompassed reverse transcription was one dispute; whether amplification must occur in a droplet was another. *Compare id.* at 31–41 *with id.* at 42–

[6] The Commission notes that Bio-Rad did not assert in response to OUII's petition that the ID's domestic industry findings would be affected by construing "amplification" to exclude reverse transcription. *See* Bio-Rad Resp. to Pets. at 34–40. To avoid confusion, however, the Commission finds that the ID's determination that 10X satisfies the domestic industry requirement is supported by a preponderance of the evidence regardless of whether "amplification" encompasses reverse transcription. This is because, as with the accused products, 10X presented unrebutted evidence that PCR in the domestic industry products satisfies the "amplification" limitation of claim 1 of the '024 patent. *See* 10X Posthearing Br. at 39 (citing CX-0004C at Q/A 278-279; CX-0481 at 11; CX-0542 at 1; CX-0579 at 1–2; CX-0578 at 15, 53). Accordingly, the Commission also affirms the ID's finding that 10X satisfied the domestic industry requirement with respect to the '024 patent.

45. The *Markman* order resolved both disputes — "amplification" is broad enough to include reverse transcription and "amplification" need not occur only in a droplet. *See* Order 22 at 32–41, 44–45.

OUII petitioned for review of the *Markman* order's conclusion on the reverse transcription issue, *see* OUII Pet. at 18–26, but no party petitioned for review of the *Markman* order's conclusion on the location of amplification issue. Bio-Rad contends that it was entitled to raise the issue in its response to OUII's petition because OUII's petition put the construction of "amplification" at issue. *See* Bio-Rad Resp. to Qs. at 31–33. That line of reasoning, if accepted, necessarily implies that by petitioning for review of one of the three issues regarding the construction of "amplification," OUII opened the door to review the other two issues as well, even though *no party petitioned for review of those issues*. Commission Rule 210.43(b)(2) provides that "[a]ny issue not raised" and "any argument not relied on" in a petition for review will be deemed abandoned. Such is the case with Bio-Rad's belated challenge to the *Markman* order's resolution of whether "amplification" must occur in a droplet. By withholding that argument until its response to OUII's petition, Bio-Rad precluded 10X and OUII from responding to that argument in their own petition responses. There would be obvious prejudice to both if the Commission declined to enforce Rule 210.43(b)(2).

Finally, the Commission notes that the noninfringement argument Bio-Rad advances in its response to the Commission's questions bears little resemblance to the argument it raised in its response to OUII's petition. Indeed, the new argument raised in Bio-Rad's response to the Commission's questions strongly suggests that even Bio-Rad understands that the noninfringement argument it raised in its response to OUII's petition is unrelated to the reverse transcription issue. For example, Bio-Rad's argument in its response to OUII's petition relied on

evidence from the *Markman* phase of this investigation to ultimately argue that "[t]he structure of claim 1 of the '024 Patent requires that amplification occur in the droplet. But 10X has presented no evidence that amplification in the Bio-Rad Accused Products (*i.e.*, PCR) occurs in the droplet and, in fact, there is evidence that this step takes place after the droplets are broken." Bio-Rad Resp. to Pets. at 39–40. The success of that argument is contingent on a claim construction that requires amplification to occur in a droplet such that the PCR in Bio-Rad's products will not read on the "amplification" limitation. As noted, Bio-Rad abandoned this argument by failing to include it in its petition for review.

By contrast, in its responses to the Commission's questions, Bio-Rad shifted its focus away from claim construction. Instead, Bio-Rad argued that the subject of the "nucleic acid amplification" limitation — "said given oligonucleotide molecule attached to said target nucleic acid analyte" — "only exists in the droplet," in Bio-Rad's products. Bio-Rad Resp. to Qs. at 29 (internal quotations omitted). That argument relies on the assumption that the target nucleic acid analyte is mRNA. *See id.* at 29–30. The argument fails to address, however, the fact that 10X did not rely solely on amplification of mRNA to satisfy the "amplification" limitation. In two of the four types of amplification 10X relied on, cDNA is the target nucleic acid analyte in both steps (b) and (c) of claim 1 of the '024 patent. *See* 10X Posthearing Br. at 24–25. As previously noted, Bio-Rad's posthearing briefing and evidence only addressed 10X's infringement allegations that relied on reverse transcription as the amplification reaction. Bio-Rad did not present evidence or argument to counter 10X's evidence and arguments that the amplification reaction is satisfied by PCR. Accordingly, the Commission finds that Bio-Rad's most recent noninfringement argument does not change the fact that a preponderance of the evidence shows that the amplification step of claim 1 of the '024 patent is satisfied regardless of whether "amplification" encompasses reverse

transcription. Moreover, because Bio-Rad raised this argument for the first time before the Commission, it is also waived. *See* 19 C.F.R. § 210.43(b)(2).

The Commission notes that Bio-Rad's response to OUII's petition for review did not argue that modifying the construction of "amplification" to exclude reverse transcription would alter the ID's conclusion that 10X satisfied the domestic industry requirement for any asserted patent, or the ID's conclusion that the '468 patent is infringed. *See* BioRad Resp. to Pets. at 39–40. Moreover, as OUII noted in its petition, 10X presented, and the ID identified, similar evidence showing amplification through PCR in the context of the domestic industry products and infringement of the '468 patent. *See* OUII Pet. at 25–26; ID at 32, 63, 66. Accordingly, the Commission also finds that whether "amplification" encompasses reverse transcription is immaterial to those issues as well.

B. Validity: Disclosure of "Porous Gel Beads" in the Prior Art

Bio-Rad petitioned for review of the ID's finding that the asserted claims of the '024 patent were not invalid as anticipated or obvious. Bio-Rad Pet. at 10–26. Like the ID, Bio-Rad's petition focused on two limitations in the asserted claims: (1) porous gel beads and (2) releasable attachment of barcodes to those gel beads. *See id.* In Bio-Rad's view, those limitations are anticipated or rendered obvious by U.S. Patent No. 9,347,059 (JX-0031, "the '059 patent") and/or U.S. Patent No. 9,902,950 (RX-0462, "the Church patent"). *See id.* On review, the Commission has determined to affirm the ID's finding that the asserted claims of the '024 patent are not invalid as anticipated or obvious with supplemented reasoning concerning the disclosure of "porous gel beads" in the prior art.

First, Bio-Rad asserted that the ID erred by relying on (1) the '059 patent's description of certain beads as "coated" and (2) the testimony of the inventor of the '059 patent that he believed he disclosed solid beads in the '059 patent to conclude that the beads were solid as opposed to

porous. *See* Bio-Rad Pet. at 10–11. However, even if those assertions of error are true, they would not provide a basis to find an affirmative disclosure of porous gel beads in the '059 patent. Bio-Rad's arguments are limited to criticizing evidence the ID relied on to support the conclusion that the antibody-linked beads are solid, *i.e.*, not porous. At best, Bio-Rad's arguments may lead to the conclusion that the composition of the antibody-linked beads is not disclosed in the '059 patent. However, Bio-Rad's arguments do not show, by clear and convincing evidence, that the antibody-linked beads of the '059 patent are disclosed as being porous.

Second, with respect to Bio-Rad's reliance on the Roche 454 sequencing technique listed in the specification of the '059 patent as disclosing the "porous gel bead" limitation, the Commission notes that neither the '059 patent itself, nor the publication by Margulies, *et al.*, cited in the '059 patent in connection with the Roche 454 sequencing technique, disclose the use of Sepharose beads with the technique. Both the '059 patent and the Margulies paper are in evidence, but neither mentions Sepharose beads. *See* JX-0031 ('059 patent); CX-1940 (Margulies, *et al.*). Rather than acknowledge this lack of disclosure, Bio-Rad represented in its petition that "[t]he undisputed testimony from 10X's expert Dr. Dear is that Margulies describes the 454 beads as being Sepharose." Bio-Rad Pet. at 11 (citing Tr. at 869:21–870:4; JX-31 at 26:52–54). However, the evidence Bio-Rad cites does not support its representation. The cited portion of Dr. Dear's evidentiary hearing testimony follows:

> Q. Now the 454, beads, those are Sepharose beads; correct?
>
> A. You mean the 454 sequencing beads?
>
> Q. That's correct.
>
> A. Yes, I believe — *at the time 454 was published, I believe they used Sepharose beads*. That's the Margulies paper. Whether they did since in their commercial instruments, I don't know. But in the Margulies paper, I believe they are Sepharose — Sepharose beads.

Tr. at 869:21–870:4 (emphasis added). Dr. Dear did not testify that the Margulies paper describes the 454 beads as being Sepharose beads. *See id.* He testified that he believed Sepharose beads were used with the technique at the time Margulies was published. *See id.* The fact that one of the expert witnesses in this investigation had a belief as to the particular type of bead used with the Roche 454 sequencing technique by the authors of the Margulies paper does not lead to the conclusion that the paper discloses the composition of those beads. Indeed, one need only review the Margulies paper, which is in evidence, to see that Margulies does not discuss Sepharose beads. *See generally* CX-1940. Moreover, Dr. Dear's testimony falls short of establishing that persons of ordinary skill in the art would understand Margulies to disclose the use of Sepharose beads. *Cf. Akamai Techs., Inc. v. Cable & Wireless Internet Servs., Inc.*, 344 F.3d 1186, 1192 (Fed. Cir. 2003) ("[T]he dispositive question regarding anticipation is whether one skilled in the art would reasonably understand or infer from the prior art reference's teaching that every claim [limitation] was disclosed in that single reference."); *Rosco v. Mirror Lite,* 304 F.3d 1373, 1380 (Fed. Cir. 2002) ("[I]f an element is not expressly disclosed in a prior art reference, the reference will still be deemed to anticipate a subsequent claim if the missing element is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill." (internal quotation marks omitted)). In addition, his testimony does not indicate that Sepharose beads must necessarily or inevitably be used with the Roche 454 technique, which would be required to show inherent disclosure. *See Akamai Techs., Inc.*, 344 F.3d at 1192 ("A claim limitation is inherent in the prior art if it is necessarily present in the prior art, not merely probably or possibly present.").

The portion of the '059 patent on which Bio-Rad relies is also inapposite to its position. The cited portion of that patent merely provides that "[i]n some embodiments, the next generation

sequencing technique is 454 sequencing (Roche) (see e.g., Margulies, M et al. (2005) *Nature* 437: 376-380)." JX-31 at 26:52–54. That statement does not support the conclusion that the Margulies publication discloses the use of Sepharose beads with the Roche 454 sequencing technique. *See id.*

Finally, the Commission notes, as did OUII, that the Roche 454 technique is a sequencing technique as opposed to the sample preparation technique that is the subject of the asserted claims. *See* OUII Resp. to Pets. at 7 (citing CX-1827C at Q/A 108–109). The ID makes that point explicitly in its discussion of the releasable attachment limitation, *see* ID at 37 (citing CX-1827C at Q/A 87, 108), but the Commission reiterates it here because it is equally applicable to the "porous gel bead" limitation. Thus, nothing in the '059 patent or the Margulies paper discloses the porous gel beads of the asserted claims. Accordingly, neither reference anticipates the asserted claims of the '024 patent, all of which include limitations drawn to porous gel beads. Similarly, neither reference can supply that limitation as part of a combination of prior art references to show that the asserted claims are obvious.

Consistent with the supplemented reasoning above, the Commission affirms the ID's finding that the porous gel bead limitation is not disclosed in the prior art. The Commission further affirms the remainder of the ID's findings with respect to the validity of the '024 patent to the extent they are not inconsistent with the reasoning herein. Those findings include that the prior art, including the Church patent, does not disclose porous gel beads with "releasably attached" oligonucleotide molecules, and that the asserted claims are not rendered obvious by a combination of prior art. Accordingly, the Commission affirms the ID's finding that no asserted claim of the '024 patent is invalid.

IV. THE '468 PATENT

The Commission determined to review all of the ID's findings related to a violation of section 337 based on the '468 patent. 84 Fed. Reg. 56835. On review, the Commission has determined to affirm with modified reasoning the ID's finding that Bio-Rad has violated section 337 based on infringement of the '468 patent. The Commission also affirms with modified reasoning the ID's findings that 10X satisfies the domestic industry requirement with respect to the '468 patent and that no asserted claim of the '468 patent is invalid. The Commission adopts the remainder of the ID's findings with respect to the '468 patent to the extent they are not inconsistent with this opinion.

For reference, claims 1 and 21 of the '468 patent follow:

> 1. A method for droplet generation, comprising:
>
> (a) providing at least 1,000,000 oligonucleotide molecules comprising barcode sequences, wherein said barcode sequences are the same sequence for said at least 1,000,000 oligonucleotide molecules, wherein said at least 1,000,000 oligonucleotide molecules are ***releasably attached*** to a bead, wherein said bead is porous;
>
> (b) ***combining said at least 1,000,000 oligonucleotide molecules and a sample comprising a nucleic acid analyte each in an aqueous phase at a first junction of two or more channels of a microfluidic device to form an aqueous mixture comprising said at least 1,000,000 oligonucleotide molecules attached to said bead and said sample***; and
>
> (c) ***generating a droplet comprising said at least 1,000,000 oligonucleotide molecules attached to said bead and said sample comprising said nucleic acid analyte by contacting said aqueous mixture with an immiscible continuous phase at a second junction of two or more channels of said microfluidic device***.
>
> * * *
>
> 21. The method of claim **1,** wherein subsequent to generating said droplet in (c), a given oligonucleotide molecule of said at least 1,000,000 oligonucleotide molecules attaches to said nucleic acid analyte, and wherein said given oligonucleotide molecule attached to said given nucleic acid analyte is subjected to ***nucleic acid amplification*** to yield a barcoded nucleic acid analyte.

'468 patent at cls. 1, 21 (emphasis added on contested limitations).

A. Construction of "Amplification" and the Effect on Infringement and Domestic Industry

As noted in the context of the '024 patent, the Commission has determined to take no position on whether "amplification" encompasses reverse transcription. As with the '024 patent, that issue is immaterial to the issue of whether Bio-Rad infringes the '468 patent and 10X satisfies the domestic industry requirement for the '468 Patent because a preponderance of the evidence shows that that "amplification" limitation is satisfied by PCR in the accused and domestic industry products even under a narrower construction of "amplification" than the one employed by the ID. *See* discussion *supra* Section III.A. Accordingly, the Commission affirms the ID's findings that the '468 patent is infringed and that 10X satisfies the domestic industry requirement for the '468 patent. *See* ID at 58–66. A preponderance of the evidence supports this finding under the construction the ID applied, as well as under a narrower construction that would exclude reverse transcription from the definition of "amplification."

B. Validity

Bio-Rad petitioned for review of the ID's finding that none of the asserted claims of the '468 patent are invalid as anticipated or obvious based on the '059 patent. *See* Bio-Rad Pet. at 33–38 The ID's finding is based on three principal findings: (1) that the "releasably attached" limitation of the asserted claims is not disclosed in the prior art; (2) that the "combining" step of the asserted claims is not disclosed in the prior art; and (3) that the "generating a droplet" limitation of the asserted claims is not disclosed in the prior art. *See* ID at 66–70. The ID also found that secondary considerations weighed against finding any of the asserted claims obvious. *See id.* at 70.

On review, the Commission has determined to affirm the ID's finding that the asserted claims of the '468 patent are not invalid, but under modified reasoning. Particularly, the Commission affirms the ID's finding that the "releasably attached" limitation in (1) above is not disclosed in the prior art and the ID's finding that secondary considerations weigh against finding the asserted claims obvious and adopts those findings in whole. *See* ID at 66, 70. Those findings, including particularly the absence of the "releasably attached" limitation from the prior art, are sufficient to support the ID's finding that the asserted claims are not invalid as anticipated or obvious by the prior art. The Commission has determined to take no position on whether the "combining" and "generating a droplet" limitations in (2) and (3) above are disclosed by the '059 patent.

V. THE '204 PATENT

The ID found that 10X failed to establish that Bio-Rad's accused products infringe any asserted claim of the '204 patent. *See* ID at 77. The ID's noninfringement finding follows from two subsidiary findings: (1) the ID found that Bio-Rad's accused products do not meet a Markush group limitation that defines the type of stimulus used to cause a capsule to release its contents; and (2) the ID found that 10X could not rely on the doctrine of equivalents to satisfy the Markush group limitation. 10X petitioned for review of the ID's noninfringement finding by challenging both findings. *See* 10X Pet. at 9–18. The Commission has determined to affirm with supplemented reasoning the ID's finding that none of the asserted claims of the '204 patent are infringed. The Commission adopts the ID's findings to the extent they are not inconsistent with this opinion.

For reference, claims 1 and 27 of the '204 patent follow:

> 1. A composition comprising a plurality of capsules, said capsules situated within droplets in an emulsion, wherein said capsules are configured to release their contents into said droplets upon the application of a stimulus to provide said contents in said droplets in said emulsion, wherein said stimulus ***is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof.***
>
> * * *
>
> 27. The composition of claim 1, wherein said contents comprise at least 10,000 barcoded oligonucleotides releasably attached to each of said capsules.

'204 patent at cls. 1, 27 (emphasis added on contested Markush group).

A. Literal Infringement

The salient issue addressed in 10X's petition is the ID's determination that Bio-Rad's products "do not literally infringe the asserted claims because they do not have a stimulus 'selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof.'" ID at 73. The crux of the ID's decision with respect to this limitation is that the stimulus that causes barcode molecules to be released in Bio-Rad's products are [redacted]. *See id.* at 74. [redacted] are not listed among the stimulus choices in the Markush group (a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof) and, therefore, Bio-Rad's products do not practice this limitation, which is incorporated into every asserted claim of the '204 patent. *See id.*

In concluding that Bio-Rad's products do not satisfy the Markush group limitation, the ID rejected several arguments from 10X. First, the ID rejected 10X's reliance on an [redacted] as the stimulus responsible for causing barcode molecules to be released from the gel beads in Bio-Rad's products. *See id.* at 74–78. The ID explained that the evidence of record did not show that an [redacted] alone would cause the release of barcode

molecules from gel beads. *See id.* at 75 ("[T]here is no evidence that the [redacted] by themselves would have any effect on the attached barcode molecules or the gel bead."). Rather, at best, 10X's evidence showed that barcode release is caused by [redacted] *See id.* ("Thus, as understood by [10X's expert,] Dr. Butte, the stimulus that causes the release of the barcode molecules from the gel bead in the accused products is the [redacted]"). Relying on the closed transition phrase "consisting of" in the Markush group, however, the ID interpreted the group to exclude additional unrecited elements, in this case, the [redacted]. *See id.* at 75–77. Thus, the ID determined that the stimulus limitation of the asserted claims could not be satisfied by the combination of an [redacted] and provision of [redacted] in Bio-Rad's products. *See id.* at 78.

The ID also rejected reliance on the [redacted] alone as the claimed stimulus. *See* ID at 77. Further to that finding, the ID noted that "there is no evidence that changing the [redacted] without the [redacted] will cause the release of barcode molecules from the gel beads." *Id.* The ID also pointed to a portion of 10X's posthearing brief that acknowledges the role of [redacted] in releasing the barcode molecules. *See id.* (citing 10X Posthearing Br. at 181–182). Regarding 10X's assertion that only the [redacted] is the claimed stimulus, the ID characterized that assertion as "unsupported attorney argument that is contradicted by the testimony of [10X's] own expert." *Id.* at 78 (citing Tr. (Butte) at 474:18-21). For these reasons, the ID found that "the accused products do not literally infringe the asserted claims." *Id.*

10X's primary argument is that an [redacted] is the claimed stimulus, and that the actions of [redacted] is the mechanism through which release is effectuated. *See* 10X

Pet. at 9–10 [redacted] is the start of a chain reaction: the [redacted]; and the contents of the capsule are released as a result. This [redacted] is applied as the trigger of a series of events leading to the release of the contents of the capsule and meets the claimed stimulus within the Markush group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof."). Relying on this premise, 10X attacks the ID from several directions, including arguing that the ID erroneously construed the claim such that the stimulus must "effectuate by itself the release of the contents without any facilitating or intermediate steps," 10X Pet. at 10, and that the ID erred by failing to give due weight to the fact that 10X stated in its various infringement contentions that only the [redacted] is the claimed stimulus, *id.* at 12–14.

None of 10X's arguments show that the ID erred in finding no literal infringement by Bio-Rad's products. First, as the ID noted, there is a pronounced lack of evidence supporting 10X's argument. For example, 10X's own expert *never* testified that an [redacted] alone was the stimulus recited in the asserted claims. Rather, Dr. Butte consistently testified that the stimulus was the [redacted]. For example, Dr. Butte testified as follows:

> Q: Sure. You're not claiming that the [redacted] is one of the claimed stimuli that's mentioned in claim 1 of the '204 patent; correct?
>
> A: It's [redacted]
>
> * * *
>
> Q: Right. But it's not the [redacted] itself; right?

A: It's not the [redacted] itself. It's the combination with the [redacted] specifically.

* * *

Q: Now, it's your view that the stimulus in the accused products is the [redacted] correct?

A: That is correct.

Tr. 371:14–18, 371:19–23, 432:13–16. Moreover, while 10X argues that the [redacted], *see* 10X Pet. at 9, its primary support for that contention is a section of equivocal corporate deposition testimony from a Bio-Rad witness who testified repeatedly that he was unsure of the purpose of [redacted] in Bio-Rad's process. *See, e.g.*, CX-0009C at 425:7–22 ("There are – there is [redacted] in that reaction. But it's required for a lot of DNA modifying enzymes. So I don't – I don't know. It's – it's not uncommon for an enzyme to bind a cofactor and – and not require additional – addition of a cofactor to – to be active. So I don't know if that – I don't know if the [redacted] that we add is – is necessary for the [redacted]."). Further still, Bio-Rad and OUII point to evidence suggesting that the [redacted] in Bio-Rad's products is unrelated to the action of the [redacted], which would directly refute 10X's argument that the [redacted]. *See* OUII Resp. to Pets. at 22; Bio-Rad Resp. to Pets. at 9–15; *see also* Tr. at 376:19–377:7, 377:11–379:4, 381:5–382:9, 383:18–384:16, 533:12–19, 564:15–565:9; JX-0050C at 56; JX-0132 at 65; RX-503C at Q/A 60–64; RX-537 at 5, RX-665C at Q/A 52, 59–65 (evidence relied on by OUII and Bio-Rad).

Second, 10X's argument that the ALJ misinterpreted its contentions about the accused stimulus is largely immaterial. *See* 10X Pet. at 12–14. Regardless of whether 10X asserted in its

briefs that only [redacted] is the claimed stimulus, the fact remains that there is little, if any, evidence to support that contention. That is, 10X's infringement argument did not fail because the ID misunderstood its contentions; it failed because those contentions do not show infringement by a preponderance of the evidence.

Finally, 10X's reliance on the word "comprising" in the preamble of the claims to argue that the presence of [redacted] in the accused products does not defeat infringement is at odds with the most analogous cases addressing the issue. Here, each of the independent claims begins with a preamble such as, "A composition comprising . . . ," '024 patent at cl. 1, "A device comprising . . . ," *id.* at cl. 23, or "A method comprising . . . ," *id.* at cl. 25. 10X relies on the word "comprising" in each to argue that the claims are open to additional unrecited elements. 10X Pet. at 11 (citing *Vivid Techs., Inc. v. Am. Sci. & Eng'g, Inc.*, 200 F.3d 795, 811 (Fed. Cir. 1999); *Northern Telecom, Inc. v. Datapoint Corp.*, 908 F.2d 931, 945 (Fed. Cir. 1990)). Based on that uncontroversial legal principle, 10X argues that "[redacted] is no different than any other *unaccused* component of the buffer that plays a role in creating the right operating environment such that the [redacted] results in release of contents." 10X Pet. at 11 (emphasis in original).

10X's argument misapprehends the ID's reasoning and fails to acknowledge the rest of the claim language. First, the ID did not find that the mere presence of [redacted] in the accused products defeated infringement. The ID found that 10X's own expert admitted that [redacted] alone did not stimulate the release of barcodes as required by the claims, but rather the [redacted] were an essential component of the stimulus. *See* ID at 75. Second, each claim uses the phrase "said stimulus is selected from the group *consisting of* . . ." in the limitation at issue. '204 patent at cls. 1, 23, 25 (emphasis added). The transitional phrase "consisting of" indicates a closed

group of elements, including only "a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof." *Id.* Because the evidence shows that [redacted] are all or part of the stimulus that caused the release of barcodes, this limitation is not met. The presence of the word "comprising" in the preamble of each claim does not negate the closed nature of the Markush group defining the set of stimuli that will read on the claim. Indeed, the cases the ID relied on to support its interpretation of the Markush group as a closed set of options dealt with exactly such claims — introduced by an open preamble with "comprising," but including a closed Markush group signaled with "consisting of." *See Multilayer Stretch Cling Film Holdings, Inc. v Berry Plastics Corp.,* 831 F.3d 1350, 1358 (Fed. Cir. 2016) (analyzing claims with "comprising" in the preamble followed by an element reciting, "selected from the group consisting of"); *Abbott Labs. v. Baxter Pharm. Prod., Inc.*, 334 F.3d 1274, 1276 (Fed. Cir. 2003) (same); *see also* ID at 74 (citing *Multilayer* and *Abbott*).

Under 10X's interpretation of the claim, the Markush group limitation would effectively become an open limitation, allowing any number of additional unrecited stimuli as long as one of the recited stimuli also had some connection to causing the capsules to release their contents. 10X cites no precedent interpreting a Markush group that introduces its elements with the signal "consisting of" in that way. To the contrary, precedent uniformly treats Markush groups using the signal "consisting of" as closed, excluding other unrecited elements absent explicit language in the claim permitting as much. *See Multilayer,* 831 F.3d at 1358; *Abbott Labs.*, 334 F.3d at 1276. Given the Federal Circuit's binding precedent, the Commission affirms the ID's reasoning that the Bio-Rad products do not infringe because the [redacted] are part of the stimulus that releases barcodes in the accused products, but the Markush group recited in the asserted claims does not encompass the [redacted]. We adopt those findings.

The Commission notes that the ID reached its conclusion without resolving the disputed issue of whether an [redacted] in the accused products. In response to the Commission's request for briefing, 10X argued that the [redacted] *See* 10X Resp. to Qs. at 28–35. In support of that argument, 10X argued that (1) [redacted]. *See id.* at 33–35. This facet of 10X's argument relied on a publication by Melamede, *et al.*, listed on the face of the [redacted] product insert.[7] *See* JX-0050C at 56; CX-1965. Particularly, 10X asserted that "Figure 6C of Melamede plots the activity of Endo VIII [redacted]. *Id.* at 34; *see also id.* at 35.

[7] The [redacted] product insert lists five articles and one U.S. Patent on its face. JX-0050C at 56. 10X relies on one of those references — Melamede, R.J., Hatahet, Z., Kow, Y.W., Ide, H. and Wallace, S.S. (1994) *Biochemistry* 33, 1255–1264 (hereinafter "Melamede") (CX-1965) — to support its argument that an [redacted] activity. Bio-Rad relies on the U.S. Patent — U.S. Patent No. 7,435,572, "Methods and Compositions for DNA Manipulation," issued to Jurate Bitinaite on October 14, 2008 (hereinafter "the '572 patent") (JX-0132) — and one of the articles — Lindhal, T., Ljungquist, S., Siegert, W., Nyberg, B. and Sperens, B. (1977) *J. Biol. Chem.* 252, 3286–3294 (hereinafter "Lindhal") (RX-0537) — to support its counter-argument that an [redacted].

10X also relies on the testimony of a Bio-Rad employee, Dr. Agresti, who provided corporate deposition testimony on behalf of Bio-Rad, and also testified at the evidentiary hearing. *See id.* at 36. Specifically, 10X notes that "Dr. Agresti provided corporate deposition testimony that [redacted], but that he did not recall which of the [redacted] required it." *Id.* In 10X's view, Dr. Agresti's deposition testimony supports its argument that "the activity [of] [sic] [redacted]." *Id.* 10X further noted that Dr. Agresti testified at the evidentiary hearing that he did not believe [redacted], but 10X characterizes that testimony as contradictory to his deposition testimony. 10X also argued that the bases of Dr. Agresti's hearing testimony — a publication by Lindhal, RX-0537, and U.S. Patent No. 7,435,572, JX-0132, both of which appear on the [redacted] product insert — were cherry-picked for him by Bio-Rad's counsel, and that neither are reliable because they concern [redacted] activity under conditions that are materially different from those found in the accused products. *See id.* at 36–40. Based on these arguments, 10X submits that a "preponderance of evidence therefore shows that an [redacted], meeting the relevant language of Claim 1 of the 204 Patent." *Id.* at 41.

Bio-Rad argued in its response that any [redacted] in the workflow of its products does not [redacted]. *See* Bio-Rad Resp. to Qs. at 34–35. Bio-Rad does not appear to dispute that [redacted] to the ddSEQ system, but submits that the purpose of that addition is to [redacted]). *See id.* at 37 ("On the contrary, the evidence shows that Bio-Rad [redacted],

[redacted].”). Bio-Rad argued that the [redacted] is already 100% active without any [redacted]. *See id.* at 35–37.

The strongest part of Bio-Rad’s counter-argument is that 10X’s cited evidence purporting to show a relationship between [redacted] is inapposite because of material differences in the conditions surrounding the experiments in the cited article and the conditions present in Bio-Rad’s products. *See id.* at 41–43. For example, Bio-Rad points out that while 10X relies heavily on Melamede, that article “tested the activity of Endonuclease VIII ***on DNA containing thymine glycols***.” Bio-Rad Resp. to Qs. at 42 (emphasis in original). Moreover, Bio-Rad submits that “Melamede expressly states that Endonuclease VIII [redacted]” *Id.* (citing CX-1965.00008). Thus, Bio-Rad argues that 10X is relying on information about [redacted] activity that is insufficiently related to the behavior of the [redacted] in the accused products. *See id.* at 41–43 (“10X does not even attempt to demonstrate that the context of Melamede has any relevance to the context of the Bio-Rad Accused Products”).

Bio-Rad also argued that 10X’s calculations of the amount of [redacted] to Bio-Rad’s products are unsupported attorney argument, and are also contradicted by witness testimony in the record. Bio-Rad Resp. to Qs. at 43–44 (citing Greiner Tr. 539:16-541:15). The point of that argument, presumably, is to further undermine any reliance on Melamede by arguing that the concentrations of [redacted] investigated in Melamede are not similar to the concentrations present in Bio-Rad’s products.

Finally, Bio-Rad pointed to the Lindhal article and the ’572 patent referenced on the [redacted] product insert as evidence that the [redacted] are either unaffected or inhibited by the [redacted]. *See id.* at 44–46; s*ee also* Bio-Rad Resp. to Pets. at 12–15.

Particularly, Bio-Rad argued that "according to Lindahl, UDG, [redacted]," Bio-Rad Resp. to Qs. at 44, and that "the '572 Patent describes the [redacted] and confirms that [redacted]," *id.* at 44–45.

OUII's response was in substantial alignment with Bio-Rad's. OUII Resp. to Qs. at 15–19. OUII reiterated the evidence it pointed to in its petition for review to show that the [redacted] used in the Bio-Rad products are active without any [redacted], that the [redacted], and that the purpose of the [redacted] present in the Bio-Rad products is to [redacted]. *See id.* at 15–18. With respect to the Melamede article, OUII takes the position that the experiments reported therein are insufficiently related to the accused products to conclude that an [redacted]. *See id.* at 18. OUII was also critical of the absence of expert testimony supporting 10X's interpretation of Melamede. *Id.*

There is no dispute that Bio-Rad's processes involve an [redacted]. There is, however, a lack of reliable evidence as to the effect, if any, that [redacted]. This is because the parties failed to show that the articles and references upon which they rely analyzed [redacted] activity in conditions that are the same or similar to those in the accused products. 10X has the burden of proving infringement by a preponderance of the evidence; the evidence does not establish that Melamede's reported relationship between [redacted] and Endo VIII's activity in nicking thymine glycols is probative of the relationship between [redacted]

[redacted] in the accused products to release barcodes. *See* Bio-Rad Reply at 35–37 (discussing evidence supporting the distinction between [redacted] in the accused products and Endonuclease VIII nicking thymine glycol). Dr. Agresti's deposition testimony is hardly persuasive on the effect of [redacted] in the accused products. When viewed in whole, the relevant portion of Dr. Agresti's deposition transcript demonstrates that Dr. Agresti did not know at the time whether the [redacted] was necessary for the [redacted] to work. *See* CX-0009C at 422:20–429:15.

Even if 10X's argument is accepted as true, it would not show that an [redacted] is the "trigger of a series of events leading to the release of" barcodes from the beads in the accused products. *Cf.* 10X Pet. at 10 (arguing that an [redacted]). According to 10X, prior to any [redacted] in the accused products, *see* 10X Pet. at 35 ("According to Melamede, that [redacted] Thus, even under 10X's theory, an [redacted] does not "trigger" the release of barcodes from beads in the accused products. The [redacted] is already active, and the presence of [redacted] only improves its activity. 10X fails to explain how that [redacted] reads onto the '204 patent's claim language requiring capsules "configured to release their contents . . . upon the application of a stimulus." *See, e.g.*, '204 patent at cl. 1. Under 10X's theory, the [redacted] will stimulate the capsules in Bio-Rad's products to release barcodes regardless of whether [redacted] is added, albeit possibly at a slower rate. Accordingly, even under its own theory of how [redacted] in the accused products, 10X

has not shown that an [redacted] is the stimulus that causes the capsules in Bio-Rad's products to release their barcodes.

In conclusion, the Commission affirms the ID's finding that 10X failed to show that the asserted claims of the '204 patent are literally infringed by the accused products.

B. Doctrine of Equivalents

Before the ALJ, 10X argued in the alternative that the Markush group limitation was satisfied by the [redacted] in the presence of a change in [redacted] ion concentration as an equivalent to the recited "reduction in disulfide bonds" element. *See* ID at 78. The ID rejected this argument, finding that 10X was estopped from relying on the doctrine of equivalents ("DOE") to satisfy this limitation. The ID's finding in that regard has two facets: (1) there is a presumption that 10X is estopped from relying on DOE based on its amendments during prosecution, *see id.* at 82; and (2) 10X had not established that its narrowing amendment was tangential to the alleged equivalent (which would overcome the presumption against DOE), *see id.* at 85.

10X petitioned for review of the ID's finding that it is estopped from relying on DOE to satisfy this element of the asserted claims. 10X does not dispute the ID's finding that a presumption of estoppel is proper, but rather faults the ID for misunderstanding what evidence was in the record.[8] 10X Pet. at 16. Particularly, 10X faults the ID's statement that "the record **is devoid of any evidence concerning Trnovsky's teachings**." *Id.* (quoting ID at 84 (emphasis 10X's)).

[8] 10X spends several pages of its petition reciting the "procedural history of Staff's [prosecution history estoppel] argument" to show "the improper burden the ID imposes on 10X." 10X, however, does not explain how the procedural history of the issue supports modifying or reversing the ID, and we find such argument meritless in any event. 10X's chief complaint appears to be that Bio-Rad raised but abandoned a similar argument, while OUII raised the argument for the first time in its prehearing brief. Presumably, 10X's implication is that it did not receive a fair opportunity to prepare evidence in response to OUII's argument. If that is the case, 10X's recourse was to seek relief from the ALJ.

10X argues this statement is clear error because Trnovsky itself is in the record, as is testimony from 10X's expert, Dr. Butte. *Id.* at 16–17.

As explained in the ID, "[d]uring the prosecution of the '204 patent, application claims 1, 78, and 110 matured into issued claims 1, 23, and 25, respectively." ID at 79 (citing JX-0009 at 13630). As originally filed, application claims 1 and 78 required a capsule(s) "configured to release their contents . . . upon the application of a stimulus," but did not require that the stimulus be selected from a particular group of stimuli. *Id.* (quoting JX-0009 at 80 (application claim 1); JX-0009 at 85 (application claim 78) (requiring a capsule "configured to release its contents into said droplets upon the application of a stimulus"). Similarly, application claim 110 required a step of "providing a stimulus to cause said capsules to release their contents into said droplets," without requiring the stimulus be selected from a group of stimuli. *Id.* (citing JX-0009 at 87).

The ID further explains that while "application claim 1 did not limit the stimulus to a group of stimuli, two of its dependent claims [(application claims 19 and 21)] did." ID at 80. Application claim 19 required the stimulus to be "selected from the group consisting of a chemical stimulus, a bulk stimulus, a biological stimulus, a light stimulus, a thermal stimulus, a magnetic stimulus, and combinations thereof," while application claim 21 required the stimulus to be "selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof." JX-0009 at 81.

A brief description of the prosecution history is helpful before addressing 10X's argument. In an office action issued on January 29, 2016, the examiner rejected all of the pending claims as anticipated in view of several prior art references. *Id.* at 9770–9781. Application claim 1 was found to be anticipated by seven references: (1) U.S. Patent Publication No. 2005/007951 to Berka et al. ("Berka"), (2) U.S. Patent Publication No. 2015/0079510 to Church et al. ("Church"), (3)

U.S. Patent Publication No. 2014.0227706 to Kato et al. ("Kato"), (4) U.S. Patent Publication No. 2003/0207260 to Trnovsky et al. ("Trnovsky"), (5) U.S. Patent Publication No. 2013/0189700 to So et al. ("So"); (6) U.S. Patent Publication No. 2004/0258701 to Dominowski et al. ("Dominowski"); and (7) U.S. Patent Publication No. 2009/0025277 to Takanashi ("Takanashi"). *Id.* at 9777–9780. Application claim 19 was rejected as anticipated by five references: (1) Berka, (2) Trnovsky, (3) So, (4) Dominowski, and (5) Takanashi. *Id.* Application claims 78 and 110 were rejected as being anticipated by Berka. *Id.* Application claim 21 was rejected as being anticipated by Kato. *Id.*

On April 28, 2016, the applicants responded to the rejections by, *inter alia*, cancelling application claims 19 and 21 and amending application claims 1, 78, and 110. As amended, application claims 1, 78, and 110 incorporated application claim 21's limitation requiring that the stimulus be "selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof." *Id*. at 10009; *see also id.* at 10000, 10002, 10003. With this amendment, the applicants argued that the amended application claims were allowable over the cited prior art with the exception of Kato. *Id*. at 10009 ("Initially, as Claim 21 was rejected only over Kato, Applicant understands that the Office acknowledges that none of Berka, Church, Trnovsky, So, Dominowski and Takanashi teach or disclose 'wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof,' as recited in claims 1, 31, 78, 89, 110 and 118."). With regard to Kato, the applicants argued that "Kato does not teach or disclose, 'wherein said capsules are configured to release their contents into said droplets upon the application of a stimulus,' as recited in Claim l." *Id.* at 10010. The applicants also argued that Kato did not qualify as prior art. *Id.*

On August 5, 2016, the examiner rejected the amended claims in view of a new set of prior art references and noted that the previous rejections had been rendered moot in view of the new grounds of rejection. *Id*. at 10074. The examiner also "noted that the 102(b) rejection of Claims 1 and 21 over Kato has been withdrawn in light of the applicant's persuasive arguments." *Id.* In response to the new rejections, the applicants further amended application claims 1, 78, and 110 to require that the capsule or capsules "provide said contents in said droplets in said emulsion" upon the application of a stimulus. *Id.* at 10118, 10120–21. The application claims as amended were allowed. *Id.* at 13617.

The Commission finds that 10X is correct that Trnovsky is in the record, and thus the ID was wrong to state that there is no record evidence of Trnovsky's teachings. Trnovsky is exhibit JX-0030, and was admitted on March 25, 2019. Tr. at 480. The ID apparently interpreted the statement in 10X's posthearing reply brief that "Staff [] did not introduce the underlying references, and the evidence of record is that they do *not* disclose [redacted] with a change in ion concentration," to mean that the Trnovsky was not introduced at all, when apparently 10X only meant that OUII did not introduce Trnovsky as an exhibit. CRB at 85; *see also* ID at 84 (citing same). Because the ID's statement concerning Trnovsky's admission is incorrect, the Commission reverses that limited portion of the ID's reasoning. However, notwithstanding that correction, 10X still has not shown why it is entitled to rely on DOE based on correction of this error.

The crux of 10X's tangential relationship argument is that Trnovsky did not disclose the combination of an enzyme with a change in ion concentration as the stimulus to cause a capsule to release its contents. 10X Pet. at 17 (quoting CX-0004C (Butte WS) at Q/A 331). Rather, the reference only disclosed the use of a specific enzyme (agarase) on its own. *See id.* Thus, 10X

argued that the amendment to overcome Trnovsky only surrendered the use of enzymes that did not work in combination with a change in pH, a change in ion concentration, or a reduction of disulfide bonds. *See id.* Thus, according to 10X, the combination of an enzyme *with* a change in pH, a change in ion concentration, or a reduction of disulfide bonds continued to be covered by the claims. *See id.*

The Commission finds that the legal support for 10X's tangential relation argument is lacking. Particularly, 10X's argument implicitly relies on the premise that the tangential relation exception to prosecution history estoppel applies if the prior art does not contain the asserted equivalents. This is incorrect. As explained by the Federal Circuit, while "[a]n amendment made to avoid prior art that contains the equivalent is not tangential," ***"[i]t does not follow [] that equivalents not within the prior art must be tangential to the amendment***." *Integrated Tech. Corp. v. Rudolph Techs., Inc.*, 734 F.3d 1352, 1358 (Fed. Cir. 2013) (emphasis added) (internal citations and quotation marks omitted). Indeed, an applicant may surrender by amendment more than what was required to overcome the prior art, and yet, the applicant cannot reclaim that excess via the DOE. *See Southwall Techs., Inc. v. Cardinal IG Co.*, 54 F.3d 1570, 1581 (Fed. Cir. 1995) ("[T]he limits imposed by prosecution history estoppel on the permissible range of equivalents can be broader than those imposed by the prior art.").

What 10X must show to rely on the tangential relation exception to prosecution history estoppel is that the reason for the applicant's "narrowing amendment was peripheral, or not directly relevant, to the alleged equivalent." *Integrated Tech. Corp. v. Rudolph Techs., Inc.*, 734 F.3d 1352, 1358 (Fed. Cir. 2013) (quoting *Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki Co.,* 344 F.3d 1359, 1369 (Fed. Cir. 2003) (en banc)). In other words, 10X must show that the reason the applicant amended the Markush group limitation to recite a change in pH, a change in ion

concentration, or a reduction of disulfide bonds was peripheral, or not directly relevant, to its alleged equivalent, *i.e.*, the action of [redacted]. That showing should "focus[] on the patentee's objectively apparent reason for the narrowing amendment, which should be discernible from the prosecution history record." *Integrated Tech. Corp.*, 734 F.3d at 1358 (internal quotation marks omitted) (quoting *Festo*, 344 F.3d at 1369).

Here, 10X has not made the required showing. Rather, 10X relies on the following testimony from its expert, Dr. Butte:

> Trnovsky did not describe [redacted] generally, but digestion with a specific enzyme: agarase (which Bio-Rad incorrectly quoted as agarose). JX-0030.00010 ([0009]). Trnovsky was overcome by the amendment because Trnovsky has no description, either in paragraph 9 or 102, which were cited by the examiner, see JX-0009.09778, of the use of agarase with a change in a change in pH, a change in ion concentration, or a reduction of disulfide bonds. One of ordinary skill in the art would understand that the amended claims no longer covered enzymes such as agarase that did not work with a change in a change in pH, a change in ion concentration, or a reduction of disulfide bonds. *However, one of ordinary skill would also understand that the claims continue to cover the use of enzymes with change in a change in pH, a change in ion concentration, or a reduction of disulfide bonds.*

10X Pet. at 17 (quoting CX-0004C at Q/A 331) (emphasis added). Even assuming that this testimony is uncontested, as 10X claims it is, it does not show that the tangential relation exception applies. Here, Dr. Butte merely testifies that the reference "Trnovsky has no description, either in paragraph 9 or 102, which were cited by the examiner, see JX-0009.09778, of the use of agarase with a change in a change in pH, a change in ion concentration, or a reduction of disulfide bonds." *Id.* But, as explained above, "[i]t does not follow [] that equivalents not within the prior art must be tangential to the amendment." *Integrated Tech. Corp. v. Rudolph Techs., Inc.*, 734 F.3d 1352, 1358 (Fed. Cir. 2013) (internal citations and quotation marks omitted).

The applicant's amendment drastically reduced the universe of stimuli covered by the Markush group to overcome an anticipation rejection based on references, such as Trnovsky, that

disclosed stimuli covered by the applicant's original, broader claims. That reason is neither peripheral nor irrelevant to 10X's alleged equivalent, which would replace a reduction in disulfide bonds with the action of [redacted] in the presence of an [redacted] ions. The action of [redacted] would have been included within the scope of the applicant's original claims, but also would have been anticipated by the disclosure of Trnovsky concerning agarase, both [redacted] and agarase enzymes being within the original Markush group consisting of a chemical stimulus, a bulk stimulus, and a biological stimulus. The applicant's amendment surrendered both enzymes by narrowing the universe of claimed stimuli drastically. Though 10X now tries to create space between the amendment's rationale and its claimed equivalent by relying on [redacted] in combination with an [redacted], it points to nothing "objectively apparent" in the prosecution history to show that the rationale for its amendment was irrelevant to enzymes in combination with an increase in ion concentrations. Particularly, Dr. Butte's testimony to that effect is wholly conclusory, and not part of the prosecution history. *See Integrated Tech. Corp.*, 734 F.3d at 1358 ("The tangential relation inquiry 'focuses on the patentee's objectively apparent reason for the narrowing amendment,' which 'should be discernible from the prosecution history record.'" (quoting *Festo*, 344 F.3d at 1369)).

At bottom, 10X's tangential relation argument against prosecution history estoppel lacks legal and evidentiary support. The ID was correct to discount it. However, the ID erroneously stated that Trnovsky is not in evidence, and that the record is devoid of evidence concerning its teachings. Accordingly, the Commission affirms the ID's finding that 10X is estopped from relying on the doctrine of equivalents to show infringement, *see* ID at 78 (finding that 10X "is precluded from relying on the DOE to satisfy the Markush group limitation."), but with the

correction that Trnovsky is in evidence and with the additional reasoning laid out above. *See* discussion *supra* pp. 35–41.

VI. THE '530 PATENT

The Commission previously determined to review all of the ID's findings related to a violation of section 337 based on the '530 patent. 84 Fed. Reg. 56835. On review, the Commission has determined to affirm with modified reasoning the ID's finding that Bio-Rad has violated section 337 based on infringement of the '530 patent. The Commission also affirms with modified reasoning the ID's finding that 10X satisfies the domestic industry requirement with respect to the '530 patent. The Commission has determined to take no position on whether Bio-Rad contributorily infringes the '530 patent. The Commission also finds that Bio-Rad abandoned the indefiniteness argument raised for the first time in its petition for review of the ID, but that even if not abandoned, the argument would fail. The Commission adopts the remainder of the ID's findings with respect to the '530 patent to the extent they are not inconsistent with this opinion.

A. Background

Of the asserted claims — claims 1, 4, 11, 14, 19, 26, 28 — claim 1 is the sole independent claim, and the bulk of the disputes with respect to the '530 patent involve the limitations recited in claim 1. All of the other asserted claims depend, both directly and indirectly, from independent claim 1. Claim 1 reads as follows:

> 1. A method for nucleic acid preparation or analysis, comprising:
>
> (a) providing:
>
> (i) at least 1,000 gel beads;
>
> (ii) releasably attached to each of said at least 1,000 gel beads, at least 1,000 barcode molecules comprising identical barcode sequences that are distinct from barcode sequences of at least 1,000 barcode molecules releasably attached to any other gel bead of said at least 1,000 gel beads; and

(iii) a plurality of cells each comprising a plurality of polynucleotide molecules;

(b) generating a plurality of droplets, wherein at least 1,000 droplets of said plurality of droplets each comprise:

(i) a single gel bead from said at least 1,000 gel beads; and

(ii) a single cell from said plurality of cells; and

(c) in each of said at least 1,000 droplets, using said plurality of polynucleotide molecules from said single cell and barcode molecules of said at least 1,000 barcode molecules from said single gel bead to generate a plurality of barcoded polynucleotide molecules,

wherein said barcode molecules become detached from said gel bead.

'530 patent at cl. 1 (emphasis added on contested limitations; indentation from "wherein said barcode molecules become detached from said gel bead" paragraph maintained from admitted joint exhibit, JX-7).

In construing claim 1, the *Markman* order rejected proposed constructions from OUII and Bio-Rad that would limit the claim by requiring that the 1,000 droplets be provided in a single experiment (Bio-Rad's proposal) or by requiring that the plurality of cells come from a common sample (OUII's proposal). *See* Order No. 22 at 46 (*Markman* Order) at 46–48. The *Markman* order also rejected 10X's argument that multiple runs of the method could be combined to reach the 1,000-droplet threshold in step (b). *See id.* at 50–51. Ultimately, the *Markman* order concluded that "claim 1 requires that the step of generating 'at least 1,000 droplets' be completed before the third step of forming a 'plurality of barcoded polynucleotide molecules' is performed in any of the droplets." *Id.* at 51.

Thereafter, on March 5, 2019, the ALJ issued Order No. 35, which denied Bio-Rad's motion for summary determination of non-infringement with respect to the '530 patent, among others things. In its motion, Bio-Rad had argued that its products did not infringe because, in them, barcoding began before all of the at least 1,000 droplets were formed. *See* Order No. 35 at 4–5.

Order No. 35 rejected Bio-Rad's argument on the basis that the *Markman* order did not interpret claim 1 such that "all 1,000 droplets form before any barcoding begins." *Id.* at 6 (internal quotation marks omitted). Rather, "[t]he claim language merely requires that any accused step of generating a plurality of barcoded molecules occurs after the at least 1,000 droplets are generated." *Id.* Order No. 35 then further explained that even if Bio-Rad's assertion were true that some barcoded molecules were formed at room temperature before the at least 1,000 droplets were generated, that would "not preclude a finding of infringement based on a subsequent step of generating barcoded molecules in a thermal cycler." *Id.* The crux of Order No. 35's reasoning is that some barcoding may occur during the droplet generation claimed in step (b) without precluding the possibility that after 1,000 droplets are generated in step (b) additional barcoding may occur that will satisfy step (c) of claim 1. *See id.* (citing *Kaneka Corp. v. Xiamen Kingdomway Group Co.*, 790 F.3d 1298, 1306, (Fed. Cir. 2015)).

The final ID reiterated and applied the claim constructions for the '530 patent from Order Nos. 22 and 35, discussed above. ID at 91.

B. "wherein said barcode molecules become detached from said gel bead."

Bio-Rad petitioned for review of the ID's findings of infringement and domestic industry with respect to the '530 patent. Among the arguments raised in Bio-Rad's petition is that neither the accused products nor the domestic industry products practice the final clause of step (c) of claim 1, which reads: ". . . wherein said barcode molecules become detached from said gel bead." '530 patent at cl. 1. Bio-Rad's arguments rely on the premise that this "wherein" clause is part of step (c), and thus subject to the ID's requirement that step (c) occur after at least 1,000 droplets are generated in step (b). In other words, barcode detachment must occur after at least 1,000 droplets are generated. There is no question that barcode detachment occurs in the accused and

domestic industry products; thus, the salient dispute raised by Bio-Rad's petition is the timing of barcode detachment.

Step (c) of claim 1, as it appears in the '530 patent, sets off the "wherein" clause with separate indentation from the other limitations of step (c). *See* '530 patent at cl. 1.[9] At the same time, the wherein clause is separated from the other clauses of step (c) with only a comma, where elsewhere in the claim separate steps are set off with semi-colons. Because the unusual indentation of the "wherein" clause raises some ambiguity as to whether that clause is part of step (c) — and thus subject to the timing requirement at the heart of Bio-Rad's argument — the Commission sought briefing from the parties on whether the "wherein" clause is included within step (c). The parties all agreed in response that the "wherein" clause is part of step (c) of the method claimed in claim 1. The Commission agrees, and therefore affirms the ID's finding that the third step of the

[9] Images from the '530 patent follow:

What is claimed is:

1. A method for nucleic acid preparation or analysis, comprising:

(a) providing:

(i) at least 1,000 gel beads;

(ii) releasably attached to each of said at least 1,000 gel beads, at least 1,000 barcode molecules comprising identical barcode sequences that are distinct from barcode sequences of at least 1,000 barcode molecules releasably attached to any other gel bead of said at least 1,000 gel beads; and

(iii) a plurality of cells each comprising a plurality of polynucleotide molecules;

(b) generating a plurality of droplets, wherein at least 1,000 droplets of said plurality of droplets each comprise:

(i) a single gel bead from said at least 1,000 gel beads; and

(ii) a single cell from said plurality of cells; and

(c) in each of said at least 1,000 droplets, using said plurality of polynucleotide molecules from said single cell and barcode molecules of said at least 1,000

* * *

US 9,856,530 B2

49

barcode molecules from said single gel bead to generate a plurality of barcoded polynucleotide molecules, wherein said barcode molecules become detached from said gel bead.

2. The method of claim **1**, wherein, prior to (c), said

50

15. The method of claim **1**, wherein, in (a), said at least 1,000 gel beads are a subset of a plurality of gel beads.

16. The method of claim **15**, wherein said plurality of gel beads comprises at least 10,000 gel beads.

17. The method of claim **1**, wherein said at least 1,000

'530 patent at cl. 1 (highlighting added on disputed clause).

claimed process "requires that the 'barcode molecules become detached from said gel bead.'" ID at 98. Accordingly, because the "wherein" clause is part of step (c), the barcode detachment required by that clause must occur after at least 1,000 droplets have been generated in step (b). The parties dispute whether the accused and domestic industry products practice the "wherein" clause so construed.

10X argued that a "preponderance of evidence shows that Bio-Rad's accused products and 10X's domestic industry products practice step (c) of Claim 1 of the ']530 Patent if the Commission finds that the barcode molecules must become detached from the gel bead during that step." 10X Resp. to Qs. at 46. Concerning the accused Bio-Rad products, 10X pointed to evidence showing that [redacted], *i.e.*, the barcodes are released during step (c). *See id.* at 46–48.

Concerning its own domestic industry products, 10X argued that "[o]n the thermal cycler in 10X's single-cell products, barcode detachment occurs and those barcodes are used to form barcoded cDNAs." *Id.* at 49. 10X further argued that "[t]he entire droplet formation process takes only several minutes, whereas 10X's technical fact witness explained upon cross-examination that the gel bead with attached barcodes persists after droplet formation." *Id.* at 50 (citing Schnall-Levin, Tr. at 224:18-23). In making that point, 10X implicitly argues that barcode release does not happen instantaneously in its products such that at least 1,000 droplets can be formed and transferred to a thermal cycler before the barcodes are released in those droplets.

By contrast, Bio-Rad argued that neither the accused nor domestic industry products satisfy the "wherein said barcode molecules become detached from said gel bead" limitation of claim 1 because in both sets of the products the barcodes become detached before a collection of at least 1,000 droplets can be generated. *See* Bio-Rad Resp. to Qs. at 54. With respect to the domestic

industry products, Bio-Rad pointed to evidence showing that [redacted] dissolves the gel beads and thus releases the barcodes immediately after droplet formation and prior to incubation on the thermal cycler. *See id.* at 58–64. Because the barcodes are released immediately after barcode formation, Bio-Rad argued that the domestic industry products do not release barcodes after at least 1,000 droplets have been formed, as required by step (b) of claim 1. Thus, Bio-Rad argued that the domestic industry products do not practice the "wherein" clause during step (c), because there is never a collection of at least 1,000 droplets in which gel beads release their barcodes. Bio-Rad also pointed out that the evidence cited in the ID to support the conclusion that barcodes are detached during incubation (and thus as part of step (c)), does not actually support that conclusion. *See id.* at 59–60. Bio-Rad further pointed to portions of the user manual cited by the ID that actually tend to show that barcodes are released prior to incubation on the thermal cycler. *Id.* at 60 (citing CX-0481 at 11).

With respect to its accused products, the crux of Bio-Rad's argument is that the [redacted]. *See id.* at 65–66. Bio-Rad disputed the ID's finding that the purpose of heating the droplets in the accused products on a thermal cycler[10] — a process that occurs after droplet formation — is to activate the [redacted]. *See id.* at 66. Bio-Rad argued that the ID incorrectly described the product label for [redacted] as describing a reaction temperature and time when the label only actually specifies a temperature. *See id.* Bio-Rad also disputed that many of its own documents cited by

[10] A thermal cycler, also known as a thermocycler, is a laboratory instrument that can be used to raise and lower the temperature of a sample in discrete, pre-programmed steps. *See* CX-0481 at 26 (10X Chromium™ Single Cell 3' Reagent Kits v2 User Guide describing three-step incubation procedure on a thermal cycler); *see also id.* at 9 (listing recommended thermal cyclers).

the ID show that [redacted]. *See id.* at 66–67. Bio-Rad also argued that the ID erred in concluding that even if the [redacted]. *See id.* at 67–68. Finally, Bio-Rad argued that the weight of expert and fact witness testimony presented supported the conclusion that the [redacted]. *See id.* at 68–70.

OUII argued, like 10X, that the ID's finding that the accused products infringe should stand under its position on the relationship between the "wherein" clause and step (c) of claim 1. OUII Resp. to Qs. at 22. OUII pointed to evidence showing that the purpose of incubating the accused products on a thermal cycler at 37°C is to [redacted]. *Id.* at 22–24. OUII thus concluded that a preponderance of the evidence shows that the accused products practice step (c) of the claimed method, including the [redacted].

OUII agreed with Bio-Rad, however, that a preponderance of the evidence does not support the conclusion that the domestic industry products practice step (c) of claim 1. Like Bio-Rad, OUII pointed to documentation produced by 10X that indicates that the gel beads in the droplets dissolve "immediately" upon droplet generation, thus releasing barcode molecules, before droplets are placed on the thermal cycler. *See id.* at 24–25 (citing CX-423C at 15; CX-0004C at Q/A 242, 260; CX-540 at 5:48–6:08).

On review, the Commission has determined to affirm, with modified reasoning, the ID's conclusion that the accused products infringe the asserted claims of the '530 patent, and affirm, with modified reasoning, the ID's conclusion that the domestic industry products practice claim 1.

1. Accused Products

With respect to the accused products, there is ample evidence to show that barcode cleavage happens on the thermal cycler when the samples are heated at 37℃ for 30 minutes. This evidence comes in the form of (1) a declaration submitted by a Bio-Rad scientist during prosecution of a Bio-Rad patent, *see* JX-0171 at 328–29 (Declaration from Bio-Rad scientist Andrew Kohlway) ("The data was generated using the protocol from the Illumina-Biorad SureCell WTA 3' Library Prep kit . . . *Droplets were incubated at 37º for 30 minutes to allow the cleaving agent to cleave the dT oligonucleotides off the bead*. Next droplets were incubated at 50ºC for 1 hour to allow cellular RNA to be reverse transcribed using dT oligonucleotide primers.") (emphasis added), and (2) Bio-Rad's own expert's testimony, *see* RX-665C at Q/A 41 ("Then another step is carried out to make sure that the [redacted] and reverse transcription reactions, which took place [redacted]. In this step, the tube with the emulsion is placed into a thermocycler that is programmed to operate at two temperatures, [redacted]. First, the thermocycler operates at 37°C (basically our body temperature) for 30 minutes [redacted].

Bio-Rad's counter arguments are unpersuasive. Bio-Rad simply lacks evidentiary support for its position that "the barcode molecules [redacted]" Bio-Rad Resp. to Qs. at 65. Bio-Rad relies heavily on the testimony of its own expert, Dr. Michael Metzker, and one of its own employees, Dr. Douglas Greiner, who testify not only that [redacted]. *See* RX-665C at Q/A 97, 102, 107; RX-507C at Q/A 65; RX-727C at Q/A 8–11, 17–20. However, as noted in the ID, Dr. Metzker's testimony stands only for the

proposition that [redacted]. *See* RX-665C at Q/A 97, 102, 107; ID at 101. That testimony does not contradict the ID's ultimate finding that the [redacted].

Dr. Greiner's initial testimony is similar, establishing only that [redacted]. *See* RX-507C at Q/A 65. Dr. Greiner's rebuttal testimony goes further and, if accepted, would establish that both [redacted]. *See* RX-727C at Q/A 8–11, 17–20. Even this rebuttal testimony, however, stops short of establishing error in the ID's finding that the [redacted]. The claimed process does not include a negative limitation precluding any [redacted] or barcoding from occurring immediately upon droplet formation. The process requires only that [redacted] and barcoding occur in at least 1,000 droplets after those droplets are generated. *See* '503 patent at cl. 1.

Moreover, Dr. Greiner's rebuttal testimony relies on the assumption that the [redacted] is active at room temperature, which is contradicted by the [redacted]. *Compare* RX-727C at Q/A 11 ("Based on my own experience, I know that enzymes generally are active at room temperature, 25°C. Also, the scientific literature shows that the [redacted]") *with* JX-0050C at 56 ("[redacted] (emphasis added)). Similarly, Dr. Greiner's testimony that [redacted] is contradicted by Bio-Rad's own reference guide, which explains that reverse transcription occurs on the thermal cycler. *Compare* RX-727C at Q/A 18 ("[redacted]

[redacted].") *with* JX-0034 at 25 ("This step reverse transcribes samples on a thermal cycler.").

Order No. 35 specifically rejected Bio-Rad's interpretation of claim 1 wherein all droplet formation must be complete before *any* barcode release and barcoding began. *See* Order 35 at 6. Under such a construction, Bio-Rad might have a stronger argument that some limited amount of barcode release and barcoding occurs before 1,000 droplets have been generated. Thus, Bio-Rad's arguments are most persuasive when viewed through the lens of a claim construction that was never adopted. While Bio-Rad now tries to adjust its argument to fit the ID's claim construction — which *does not* require *all* droplet generation to be complete before any barcodes are released — the two are an imperfect match, which leads to Bio-Rad's failure on this issue.

At bottom, the dispute here is a factual one about the operation of Bio-Rad's products. The ID considered this dispute, including the testimonial evidence from Bio-Rad's expert, and concluded that "10X has shown by the preponderance of the evidence that at least the bulk of the following processes occur while the droplets are being heated on the thermal cycler: (1) the [redacted] release the barcode molecules from the gel bead and (2) the reverse transcription of barcoded cDNA from mRNA and barcode molecules." ID at 102. The Commission has determined to affirm that ultimate finding under the modified reasoning given above.[11]

[11] The ID misstates a piece of evidence on which it relies to reach that conclusion. Particularly, the ID describes exhibit JX-0050C at 56, which is a picture of the [redacted] product label, as "[redacted]" ID at 100. However, the label reproduced on the exhibit does not state that incubation should occur for 30 minutes. Instead, it states as follows: "[redacted]" JX-0050C at 56.

Bio-Rad pointed out this discrepancy in its petition for review, *see* Bio-Rad Pet. at 61, and neither OUII nor 10X disputed the point. To the contrary, 10X's response to Bio-Rad's petition is carefully worded to avoid misrepresenting the [redacted] product label. *See* 10X Resp. to Bio-Rad Pet. at 65 ("The ALJ relied upon Bio-Rad's documentation that shows the RT program at the thermal cycler contains a step of incubating the droplets at 37°C for 30 minutes, which

2. Domestic Industry Products

Turning to the domestic industry products, although the ID found that "[w]hile the droplets are being heated on the thermal cycler, the barcode molecules are released from the gel bead through the application of [redacted], which dissolves the disulfide bonds holding the barcode molecules to the gel beads," the exhibits that were cited to support that statement do not, on their face, support it. ID at 115 (citing CX-0481.0 at 11; CX-0004C (Butte DWS) at Q/A 481). Page 11 of CX-0481 (10X's Single Cell 3' Reagent Kits v2 User Guide) says nothing about barcode molecules being released from a gel bead during incubation on a thermal cycler. CX-0481 at 11. Rather, that exhibit describes incubation as occurring *after* dissolution of the gel bead delivering the barcodes. *See id.* That evidence does not address whether barcodes are released in the domestic industry products after at least 1,000 droplets have been generated as required by step (b) of the asserted claims.

Further, Q/A 481 of CX-0004C, Dr. Butte's witness statement, relates to infringement by Bio-Rad's accused products, not 10X's domestic industry products. CX-0004C at Q/A 481. Though no party petitioned for correction, this citation in the ID appears to be an inadvertent error. However, even assuming that the citation is an oversight, the portions of Dr. Butte's witness statement that *are* directed to domestic industry still do not support the conclusion that barcodes are released on the thermal cycler. *See id.* at Q/A 580–81.[12]

---

matches the reaction *temperature* for [redacted] as shown in [redacted] product label." (emphasis added)). Accordingly, the Commission has determined to modify the sentence starting on the seventh line of page 100 of the ID to read [redacted] Notwithstanding this modification, the Commission nonetheless agrees with and affirms the ID's conclusion that the accused products practice step (c) of claim 1.

[12] The parties addressed waiver at length in their responses to the Commission's request for briefing on whether the domestic industry products practice the "wherein" clause limitation of step

Nevertheless, the Commission has determined that, more likely than not, barcodes are still being released in the domestic industry products after at least 1,000 droplets have been generated, thus satisfying step (c) in combination with the ID's finding that barcoding of the polynucleotide molecules occurs on the thermal cycler in the domestic industry products. *See* ID at 115–16; *see also* CX-0481 at 11; CX-0004C at Q/A 576–78. Particularly, while evidence identified by Bio-Rad and OUII does establish that some of 10X's promotional materials explain that the gel bead dissolves "immediately" after droplet generation, *see* CX-423C at 15; CX-540 at 5:48–6:08; RX-665C at Q/A 116, counter-evidence identified by 10X shows that while the process may begin immediately, gel bead dissolution is not instantaneous, and that when at least the last 1,000 droplets are formed in the domestic industry products, dissolution of the gel beads in those droplets will not yet have occurred, but will occur shortly thereafter. *See* CX-0076C at 36; CX-0116C at 27; *see also* 10X Reply at 50–53 (citing same).

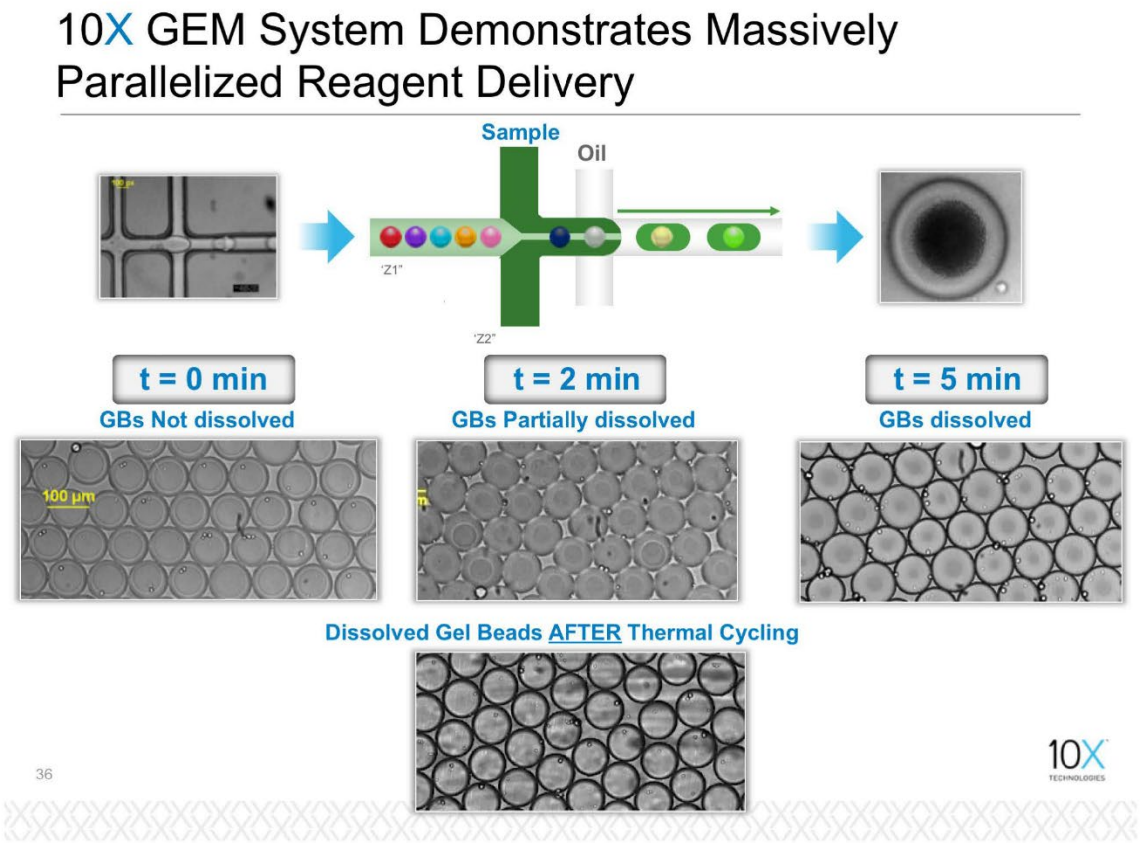
10X's counter-evidence establishes two main points in support of its position. First, it establishes that, if used according to 10X's recommendations, 17,000 cells are loaded into each of eight reaction lanes on a 10X chip, which results in recovery of about 8,000 droplets each with one gel bead and one cell. *See* CX-0004C at Q/A 570; CX-0481 at 15; *see also* 10X Reply at 50 (citing same). Because a typical run of droplet formation lasts approximately 6.5 minutes, more than 1,000 droplets are generated just in the last minute of the droplet formation process. *See* CX-0481 at 13, 23 (describing ~6.5 minute run time); 10X Reply at 51–52 ("Taking the example described above of loading a small number of cells per channel to generate 8,000 good droplets over a six

(c). *See* 10X Reply at 39; OUII Resp. to Qs. at 24, 24 n.12; OUII Reply at 19 n.14; Bio-Rad Resp. to Qs. at 54 n.9; Bio-Rad Reply at 48–50. The parties fail to acknowledge that the Commission enjoys *sua sponte* authority to review any aspect of an ID. *See* 19 C.F.R. § 210.44. Here, where the evidence cited by the ID does not support the ID's finding, such *sua sponte* review is appropriate.

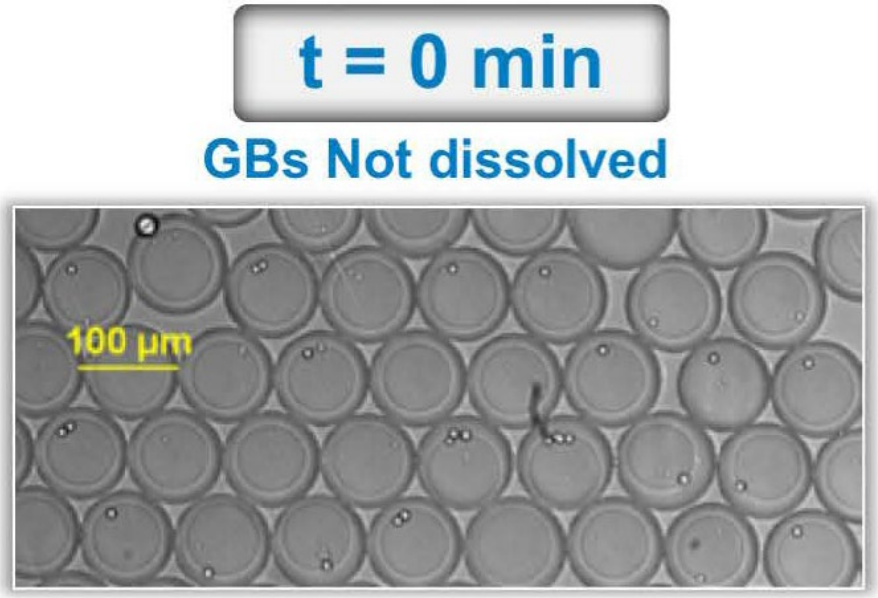
minute run (*see* CX-0477.00002) means that at least 1,000 good droplets are generated in the last minute alone of droplet formation." (footnote omitted)). The crucial question then is whether those droplets generated in the last minute still contain gel beads with attached barcodes. If they do, then the release of those barcodes will satisfy the "wherein" clause of step (c) of the claimed method. If, however, the gel beads dissolve instantaneously as each droplet is formed, the "wherein" clause of step (c) would not be satisfied because, per the construction of this claim, step (c) must occur after at least 1,000 droplets have been generated in step (b).[13]

The second point established by 10X's counter-evidence addresses that crucial question. The evidence shows that the gel beads in 10X's domestic industry products are only partially dissolved two (2) minutes after droplet formation. *See* CX-0076C at 36; CX-0116C at 27; *see also* 10X Reply at 52 (citing same). The following slide, which appears in two of 10X's investment presentations admitted into evidence, is illustrative:

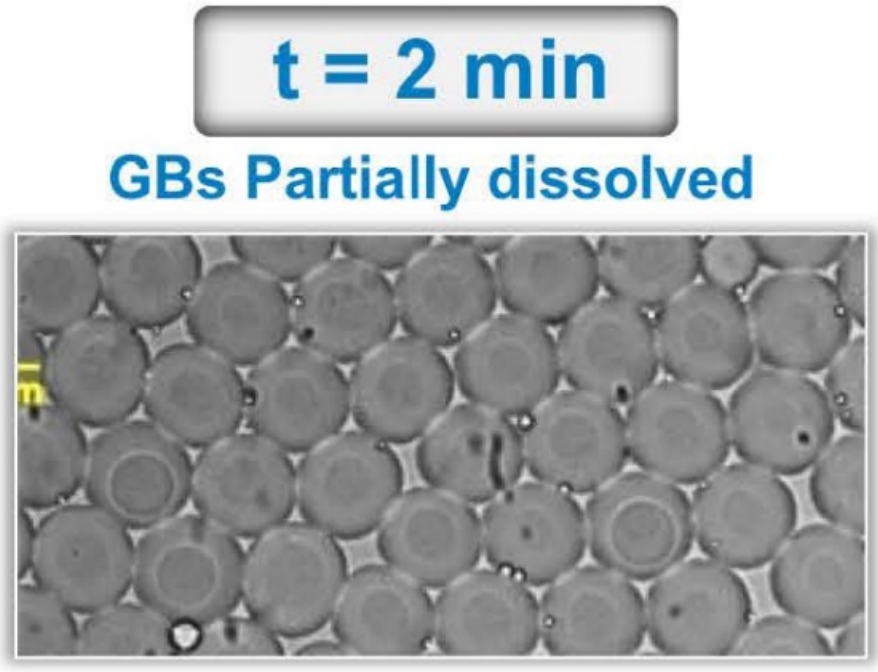
[13] The claim requires that a generated droplet must contain within it both a single gel bead with barcodes attached and a single cell made up of polynucleotide molecules. *See* '530 patent at cl. 1 (steps (a) and (b)). Inside the droplet, barcodes are released from the gel bead and then combine with the polynucleotide molecules to form barcoded polynucleotide molecules. *See id.* (step (c)). There is no dispute that all of this occurs in each droplet generated in the domestic industry products. *See, e.g.*, Bio-Rad Pet. at 63 (acknowledging formation of barcoded polynucleotide molecules in droplets in the domestic industry products). The dispute between the parties is over the timing of this process. *See, e.g.*, *id.* at 63–65.



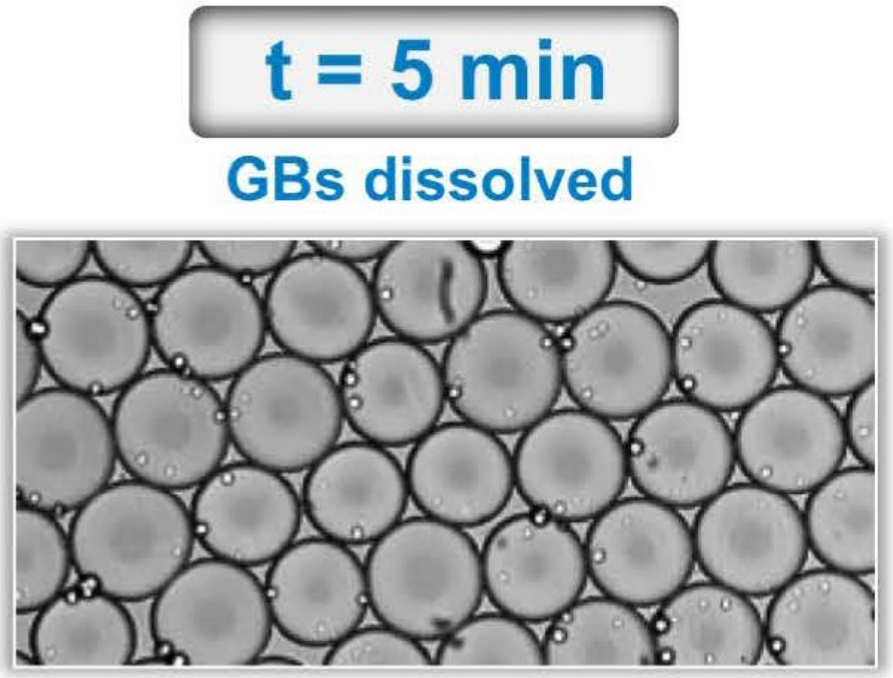
CX-0076C at 36; *see also* CX-0116C at 27 (same image in black and white). The image on the left of the middle row shows that immediately after droplet formation (t=0 min), the gel beads inside the droplet have a defined, circular boundary:



Id.; *see also id.* at 23 (illustrating components of droplet containing a gel bead). At two (2) minutes after droplet formation (t=2 min), the image in the center of the middle row shows gel beads with a blurred boundary, which are described as "partially dissolved":



See CX-0076C at 36. And, at five (5) minutes after droplet formation (t=5 min), the image on the right of the middle row shows droplets with no visible boundary around a gel bead, which are described as "dissolved":



See id. Accordingly, the Commission agrees that "whatever 'immediately' means in 10X's promotional literature, it does not mean that [redacted] dissolves the gel beads so fast that fewer than 1,000 of them still have barcodes attached after the completion of droplet formation." 10X Reply at 52.

The Commission also agrees that this evidence adequately addresses OUII's and Bio-Rad's argument that the use of the word "immediately" in 10X's promotional material means that all barcodes were released instantaneously after droplet formation. 10X's evidence is also consistent with the testimony of Dr. Schnall-Levin, who testified on cross-examination that the gel bead does not disappear instantaneously after droplet formation:

Q. When you take the first droplet, the cell and bead disappear immediately; right?

A. No, I don't think so.

Tr. at 224:18–23. Accordingly, the Commission has determined to affirm under modified reasoning the ID's finding that 10X satisfied the domestic industry requirement with respect to the '530 patent.

C. Infringement of Dependent Claim 26

Dependent claim 26 requires that the gel beads have at least 1,000,000 barcode molecules. '530 patent at cl. 26 ("26. The method of claim 1, wherein said at least 1,000 barcode molecules are at least 1,000,000 barcode molecules."). The ID found that "the WTA 3' v1, [redacted] and scATAC-seq assays infringe claim 26." ID at 105.

10X and OUII both petitioned for review of the ID's finding that dependent claim 26 of the '530 patent is infringed by the accused products. *See* 10X Pet. at 19; OUII Pet. at 17. Particularly, both argued that the ID inadvertently omitted the [redacted] from the list of infringing assays for claim 26. *See* 10X Pet. at 19; OUII Pet. at 17. Bio-Rad did not dispute 10X and OUII's position in its response to their petitions for review. *See generally* Bio-Rad Resp. to Pets.

Upon review of the ID, we agree with 10X and OUII that the omission of the [redacted] in the portion of the ID listing the assays that infringe dependent claim 26 of the '530 patent is the result of a clerical error and should be corrected. *Cf.* ID at 105. Where the ID excluded an assay from its infringement findings, it did so explicitly and with an explanation, as in the case of claim 4. *See id.* at 103. However, in the ID's analysis of claim 26, there is no discussion of the [redacted] specifically. *See id.* at 105. Moreover, the record shows that 10X timely submitted evidence to establish infringement of claim 26 with respect to all four assays. CX-

0004C at Q/A 554–556. Accordingly, the Commission has determined to modify the ID's findings to include the [redacted] among the assays that infringe claim 26.

D. Contributory Infringement

OUII petitioned for review of the ID's finding that "10X has failed to show that using the scATAC-seq assay with isolated nuclei is not a substantial non-infringing use of the ddSEQ vl products," ID at 112, which defeated 10X's allegations of contributory infringement with respect to the '530 patent. *See* OUII Pet. at 17–18. In OUII's view, the finding should be reversed because "as of the time of the hearing, the record evidence showed a lack of substantial, non-infringing uses for the ddSEQ v1 products under the '530 patent." *Id.* at 18. OUII noted, however, that even if the ID's finding was reversed, the ID's ultimate finding of violation would not be affected because the ID found that Bio-Rad induced infringement of the '530 patent. 10X summarily joined OUII on this issue in its response to OUII's petition for review. *See* 10X Resp. to OUII Pet. at 7. Bio-Rad did not respond to OUII's petition on this issue. *See generally* Bio-Rad Resp. to Pets.

The Commission has determined to take no position on whether 10X has established contributory infringement with respect to the '530 patent. The Commission affirms the remainder of the ID's findings with respect to indirect infringement of the '530 patent, including specifically its finding that Bio-Rad induced infringement of the '530 patent.

E. Indefiniteness

The Commission asked the parties to brief whether "any party argue[d] in its pre- or post-hearing briefing that the ALJ's construction of claim 1 of the '530 patent, as laid out in orders 22 and 35, was indefinite." Notice at 4. No party contended in response that indefiniteness was briefed in either pre- or post-hearing briefing. Bio-Rad and OUII, nonetheless, argued that Bio-Rad's indefiniteness argument is not waived. Notably, Bio-Rad and OUII adopted different rationales for why waiver does not apply.

OUII pointed back to Bio-Rad's briefing during the *Markman* stage of the hearing, where Bio-Rad argued that claim 1 of the '530 patent was indefinite. *See* OUII Resp. to Qs. at 26. The *Markman* order rejected that indefiniteness argument on the basis that Bio-Rad had conflated breadth with indefiniteness. *See* Order No. No. 22 at 46. OUII submitted that because the "*Markman* Order rejected Bio-Rad's indefiniteness arguments in view of the 'clear and readily understood' meaning of the disputed terms," it also "implicitly h[eld] that the Order's own construction did not render the claim indefinite." *Id.* OUII further submitted that an instruction in the *Markman* order directing the parties' subsequent briefing to apply the *Markman* order's constructions "presumably limit[ed] the parties to challenging the ordered constructions in petitions for review." *Id.* (citing Order No. 22 at 52 ("Hereafter, discovery and briefing in this Investigation shall be governed by the construction of the claim terms in this Order.").

Bio-Rad did not point to its *Markman* stage indefiniteness argument to avoid waiver. Instead, Bio-Rad argued it was precluded from raising its indefiniteness argument by the timing of Order Nos. 22 and 35. Bio-Rad Resp. to Qs. at 70–71. Expanding on that idea, Bio-Rad explained that it "believed that, as a result of the limitations imposed on the claimed method in the *Markman* Order, in particular, the requirement that step (b) of the method be completed in all 1,000 droplets before step (c) was performed on any of the droplets, a requirement the judge identified in finding the claim definite, it no longer had a basis to argue indefiniteness in its Prehearing Brief, as it had previously argued during claim construction." *Id.* at 71. Bio-Rad appears to have argued though that Order No. 35, which clarified the construction of claim 1 given in the *Markman* Order, either gave rise to a new basis for arguing indefiniteness or revived its prior basis. *See id.* at 72. Bio-Rad's briefing also suggested that the language of the *Markman* Order directing the parties to

apply the constructions therein precluded it from raising its indefiniteness arguments. Bio-Rad Reply at 53.

On review, the Commission has determined that the indefiniteness challenge raised by Bio-Rad in its petition for review is new, could have been presented before the ALJ, was not presented before the ALJ, and therefore is waived. *See* Ground Rule 11.1.

If OUII were correct that Bio-Rad's indefiniteness arguments before the ALJ during the *Markman* phase of the investigation preserved the indefiniteness arguments in its petition, Bio-Rad would, presumably, be limited to challenging the *Markman* Order's resolution of Bio-Rad's indefiniteness argument. Bio-Rad's petition is, however, silent on the reasoning given in the *Markman* Order rejecting Bio-Rad's indefiniteness argument at the time. *See* Bio-Rad Pet. at 48–55. The *Markman* order explained that:

> Bio-Rad asserts that the terms "providing," "plurality of cells," and "at least 1,000 droplets" render the claim indefinite because the claim "calls for the generation of 1,000 droplets containing specific material but does not describe how or under what circumstances those droplets are formed." RRB at 23. In making this argument, Bio-Rad confuses breadth with indefiniteness. Breadth does not render a claim indefinite. *BASF Corp. v. Johnson Matthey Inc.*, 875 F.3d 1360, 1367 (Fed. Cir. 2017 ("[B]readth is not indefiniteness.") (quoting *SmithKline Beecham Corp. v. Apotex Corp.*, 403 F.3d 1331, 1341 (Fed. Cir. 2005)) (internal quotation marks omitted); Manual of Patent Examining Procedure § 2173.02 ("A broad claim is not indefinite merely because it encompasses a wide scope of subject matter provided the scope is clearly defined"). Standing alone and in the context of the claim, the claim terms identified by Bio-Rad are clear and readily understood "even to lay judges." *Phillips*, 415 F.3d at 1314. Based on the foregoing, I find that Bio-Rad has not shown that claim 1 is indefinite.

Order No. 22 at 46. Bio-Rad's petition did not address the *Markman* Order's conclusion that Bio-Rad mistook breadth for indefiniteness. Instead, Bio-Rad's petition argued that "[t]he ID construction renders the claim indefinite both because it permits aggregation of multiple runs and because it eliminates the requirement that the method steps be performed in a specific order." Bio-Rad Pet. at 48. Moreover, Bio-Rad's petition made clear that the indefiniteness argument raised

therein is based on the construction applied in the ID, which, in Bio-Rad's view, is consistent with the clarified construction of Order No. 35, but not with the construction in the *Markman* Order. *See* Bio-Rad Pet. at 48 ("*The ID construction renders the claim indefinite* both because it permits aggregation of multiple runs and because it eliminates the requirement that the method steps be performed in a specific order." (emphasis added)). Bio-Rad's focus on the clarified construction of Order No. 35 suggests that Bio-Rad itself does not view its *Markman* indefiniteness argument and its petition indefiniteness argument as one and the same. Moreover, Bio-Rad's focus on the timing of Order No. 35, *i.e.*, that it was issued after Bio-Rad submitted its prehearing brief, as a reason it could not raise its indefiniteness argument at the hearing or in post-hearing briefing further supports the conclusion that the indefiniteness argument in the petition is distinct from the one raised before the ALJ. If not, the timing of Order No. 35 would be irrelevant, as Bio-Rad would have already had the opportunity to raise its indefiniteness argument during the *Markman* proceeding. Put differently, by arguing unfairness in the timing of Order No. 35 to support raising indefiniteness on review, Bio-Rad effectively undercut any argument that its petition's indefiniteness argument was preserved by its *Markman* indefiniteness argument.

Moreover, the indefiniteness argument in Bio-Rad's petition included new arguments that it did not raise in its *Markman* briefing. During the *Markman* process, Bio-Rad relied exclusively on the fact that the claims did not specify whether the droplets had to be generated in a single experiment or in multiple experiments. Bio-Rad Opening Markman Br. at 31 ("Nothing in the intrinsic evidence clarifies how or when the claimed 1,000 droplets each containing a gel bead and a cell should be generated. For example, the droplets could be generated in one experiment or in multiple experiments."). By contrast, the indefiniteness argument in Bio-Rad's petition is based on the theories that "numerical limitations in method claims must be met in each run of the method,

and cannot be met through aggregation of multiple runs," Bio-Rad Pet. at 48, and "[i]f the '530 Patent encompasses a continuous process, the '530 Patent is indefinite because the plain language of the claims does not inform a person of skill in the art with reasonable certainty about the scope of the claimed method." *Id.* at 54–55. Even assuming that the multiple experiment argument of the *Markman* brief and the aggregation argument of the petition are the same — an assumption which is not clearly justified — the continuous-process argument is still a new theory of indefiniteness that was never presented to the ALJ.

In a similar vein, the indefiniteness argument in Bio-Rad's petition relies on new evidence that was never presented to the ALJ in connection with indefiniteness. Particularly, Bio-Rad relies on deposition testimony from one of the inventors of the '530 patent and a 10X executive (Dr. Michael Schnall-Levin) to support its petition's indefiniteness argument. *See* Bio-Rad Pet. at 52. Bio-Rad did not rely on testimony from Dr. Schnall-Levin in its *Markman* briefing.

At bottom, the indefiniteness argument raised in Bio-Rad's petition is a new argument that was never raised before the ALJ. The Commission does not agree with OUII that the instruction in Order No. 22 requiring the parties to apply the constructions therein precluded the parties from asserting the indefiniteness of those claims as construed. A more reasonable reading of that statement is that the parties should not present multiple analyses based on different claim constructions going forward in the case.

Bio-Rad's argument that it has not waived its petition's indefiniteness arguments because the timing of Order No. 35 prevented it from raising the argument at the hearing or in its briefing is not persuasive. First, the argument is premised on Bio-Rad's belief that Order No. 35 reversed the construction of claim 1 given in Order No. 22. The Commission does not agree, however, that the two orders are inconsistent with each other. Rather, Bio-Rad interpreted Order No. 22 in a

way that was not correct — it interpreted the order such that any barcoding that occurred prior to the completion of droplet formation would defeat infringement — and Order No. 35 pointed out as much in denying Bio-Rad's motion for summary determination of no infringement. Bio-Rad's misinterpretation of Order No. 22 cannot be a reason to excuse its failure to argue indefiniteness before the ALJ.

However, even if Order No. 35 *had* materially altered the construction of claim 1 of the '530 patent, Bio-Rad's late indefiniteness argument would still be waived. This is because Bio-Rad could have sought relief from the ALJ, but did not. For example, Bio-Rad could have asked the ALJ for leave to amend its prehearing filings on the basis that Order No. 35 provided a new construction that it could not possibly have addressed in those filings. But Bio-Rad did not seek such leave. Instead, it waited until after the ID issued to argue that the clarification given in Order No. 35 rendered claim 1 indefinite. That course of action prevented 10X and OUII from developing testimony or introducing evidence to rebut that argument, and prevented the ALJ from considering the argument. While Bio-Rad argues repeatedly that it was "denied the opportunity" to argue that the ALJ's construction of claim 1 was indefinite, there is no support for that statement. Bio-Rad Reply at 53. Particularly, it is not clear why Order No. 22's statement that "[h]ereafter, discovery and briefing in this Investigation shall be governed by the construction of the claim terms in this Order," would preclude Bio-Rad from arguing that claim 1 was indefinite. If Bio-Rad had sought leave to raise its indefiniteness argument at the hearing after receiving Order No. 35, and if the ALJ denied that request, Bio-Rad would be on much stronger ground to argue that it was not permitted to make its indefiniteness argument. That is not what happened though. Bio-Rad simply did not argue that claim 1 as construed was indefinite until after the ID issued. That is waiver.

In the alternative, even if there were no waiver, Bio-Rad has not shown by clear and convincing evidence that claim 1 of the '530 patent is indefinite. *See BASF Corp. v. Johnson Matthey Inc.*, 875 F.3d 1360, 1365 (Fed. Cir. 2017) (explaining that the defendant has "the burden of proving indefiniteness by clear and convincing evidence."). Concerning the argument it made at the *Markman* phase of the investigation, the Commission agrees with the ALJ's reasoning in Order No. 22 that Bio-Rad's arguments conflated broad claims with indefinite ones. The fact that the claim does not limit droplet generation to one particular mode, *i.e.*, in a single experiment, or from a single sample, or in one run, etc., simply means the claim is broad and all of those modes are covered. Bio-Rad cannot manufacture uncertainty in the claim by arguing that only one mode can be claimed and then arguing that the claims fail to specify the particular mode.

Bio-Rad's petition-stage indefiniteness argument fails for multiple reasons. First, the argument is based on Bio-Rad's continued misinterpretation of the ID's construction of the claim. Bio-Rad argued that the ID's construction of claim 1 allows aggregation of multiple runs to meet the numerical limitations therein. Explaining that assertion, Bio-Rad argued that because its chips each have four lanes, processing droplets on one chip is actually four different experimental runs. Because the ID found that a chip generates approximately 1,200 droplets, Bio-Rad argued that the ID relied on the aggregation of four different runs that each generate about 300 droplets to find infringement. *See* Bio-Rad Pet. at 49. Bio-Rad relies on *Applera Corp. v. Illumina, Inc*., 375 Fed. App'x. 12, 20-21 (Fed. Cir. 2010), and *In re Varma*, 816 F.3d 1352, 1362–64 (Fed. Cir. 2016), for the proposition that aggregation is not permitted.

The Commission disagrees with Bio-Rad's aggregation argument because nothing in the claim indicates that the method must be confined to a single lane on a chip. *See* '530 patent at cl. 1. To the contrary, the specification clearly contemplates that different machinery used together

can practice the invention. *See* '530 patent at 10:1–18 (describing use of a device with microwell chambers to practice the method). Further, the concerns animating *In re Varma* and *Applera* are not present here. The portion of *In re Varma* relied on by Bio-Rad simply stands for the proposition that where a claim recites an object that performs two functions, the claim is not practiced by two objects that each perform one of the functions. *In re Varma*, 816 F.3d at 1363 ("For a dog owner to have 'a dog that rolls over and fetches sticks,' it does not suffice that he have two dogs, each able to perform just one of the tasks."). That issue is not present here where the claims do not include a requirement that a single lane on the chip generate at least 1,000 droplets.

Applera is no more on point. There, the claim at issue, in simple terms, covered a three-step process where the third step was to repeat the first two. *Applera*, 375 Fed. App'x at 20. The patentee advanced a construction that would allow one to skip the second step of the process for some repetitions of the process. The Federal Circuit agreed with the district court that such a construction was incorrect because it abrogated the second step of the process. *Id.* at 20–21. Thus, neither *Applera* nor *In re Varma* stand for a broad prohibition on aggregation as Bio-Rad contends. The Commission further notes that neither of those cases addresses indefiniteness based on aggregation.

Separate from *Applera* and *In re Varma*, Bio-Rad argued that if aggregation is permitted, claim 1 is indefinite because "there is no starting point and no endpoint that defines any particular method cycle" and "[a]ny number of droplets containing a single bead and a single cell, with reagents for barcoding, can be generated at any time over the course of any number of runs, on any number of independent droplet generators." Bio-Rad Pet. at 50. Bio-Rad then argued that "[a]s long as, at some point, it is determined that at least 1,000 productive droplets were generated where barcoding occurred, the limitations of the claim are met," and submits that such a claim is

in conflict with *Nautilus, Inc. v. Biosig Instruments, Inc.*, 572 U.S. 898 (2014). Bio-Rad relied on *Dow Chemical Co. v. Nova Chemicals Corp*., 803 F.3d 620 (Fed. Cir. 2015), and *Icon Health & Fitness, Inc. v. Polar Electric Oy*, 656 Fed. Appx. 1008 (Fed. Cir. 2016), as analogous situations where indefiniteness was found. Bio-Rad at 50. Bio-Rad also argued that deposition testimony from 10X's expert and an inventor of the '530 patent indicates that claim 1 has no objective boundaries. Bio-Rad Pet. at 51.

First, Bio-Rad's assertions that claim 1 has no starting point or end point under the ID's constructions are baseless. Claim 1 has three steps: (a) a "providing" step in which raw materials are provided; (b) a "generating" step in which those raw materials are used to generate droplets; and (c) a barcoding step where barcoded polynucleotides are generated in at least 1,000 droplets. '530 patent at claim 1. The claimed method starts at the providing step and ends after barcoding has occurred in at least 1,000 droplets. *Id.* Bio-Rad's argument attempts to manufacture uncertainty in an otherwise straightforward three-step claim by focusing on limitations that are not present in the claim — for example, that droplets must be generated in a single "run," or that they must be generated only in a single droplet generator, or only in droplet generators that are not independent. *Cf.* Bio-Rad Pet. 50. Bio-Rad's indefiniteness argument is not directed at claim 1 of the '530 patent; it is directed at a claim of its own making, *i.e.*, a strawman.

The cases Bio-Rad relies on bear little resemblance to the facts in this investigation and are of little relevance. *Dow* dealt with the claim phrase "slope of strain hardening coefficient greater than or equal to 1.3," which the facts in that case showed could be calculated four different ways — each with different results. *Dow Chemical Co.*, 803 F.3d at 631–634. This investigation does not present that scenario, nor even an analogous scenario. *Icon Fitness* found a claim indefinite where the evidence of record showed that the terms "in-band" and "out-of-band" were relative

terms that only have meaning in the context of a defined reference. *Icon Health & Fitness, Inc.*, 656 Fed. App'x at 1016. Here again, that scenario is not presented in this investigation. And, with respect to *Nautilus*, a case that dealt with the meaning of the phrase "spaced relationship" in exercise equipment, *see Nautilus*, 572 U.S. at 903–906, but which is legally significant for striking down the Federal Circuit's prior formulations of the test for indefiniteness, *see id.* at 901, Bio-Rad relies on the case for broad assertions unrelated to the facts of *Nautilus*. This includes the assertion that "the fact that the ALJ issued and applied two conflicting constructions over the course of the investigation supports the indefiniteness of the '530 Patent claims," Bio-Rad Pet. at 38–39 (citing *Nautilus*), and that open ended claims "violate[] the strictures of *Nautilus*," *id.* at 50. Yet, Bio-Rad's reliance on *Nautilus* is little more than a collection of unsupported assertions that the ID's construction of claim 1 somehow conflicts with the reasonable certainty standard for indefiniteness laid out in *Nautilus.* Merely identifying the case that lays out the standard for indefiniteness and then asserting that the standard is met, or not met, is not clear and convincing evidence of invalidity, which is what is required.

The expert testimony Bio-Rad relies on does not meet its burden either. *See* Bio-Rad Pet. at 51. The citations from the transcript of Dr. Butte's deposition show the attorney and Dr. Butte having a lengthy discussion about what is and is not a "common process," with Dr. Butte giving, admittedly, widely varying answers. *See* JX-157 at 123:13–137:3. Bio-Rad relied on this testimony to argue that whether aggregation is permitted depends on the vagaries of a person's opinion, thus rendering claim 1 indefinite. *See* Bio-Rad Pet. at 51–52. This entire line of reasoning is tainted however by the fact that, again, there is no limitation in the claim requiring droplet generation to occur on a single machine, in a single experiment, as part of a single "run," from a single "sample," or as part of a "common process." *See generally* '530 patent at cl. 1. An expert's

extrinsic testimony on a limitation that is not present in the claims is not probative evidence of indefiniteness. For that reason, we also find Bio-Rad's reliance on *Interval Licensing LLC v. AOL, Inc.*, 766 F.3d 1364, 1371 (Fed. Cir. 2014), which found the term "unobtrusive manner" depended on a person's subjective opinion and therefore rendered the claim in which it appeared indefinite, to be inapposite. *See* Bio-Rad Pet. at 51–52. *Teva Pharm. USA, Inc. v. Sandoz, Inc.*, 789 F.3d 1335, 1345 (Fed. Cir. 2015), which Bio-Rad also relies on in connection with Dr. Butte's testimony, is also unhelpful as the indefiniteness issue in *Teva* is essentially identical to the one in *Dow*. *See* Bio-Rad Pet. at 52.

Bio-Rad's reliance on Dr. Schnall-Levin's deposition testimony is no more probative. *See id.* (citing RX-413C at 285:19–24). Bio-Rad asked Dr. Schnall-Levin if the patent provided directions of how many cells to run per chip in claim 1, and Dr. Schnall-Levin answered that there were no instructions on cells per chip. *See id.* This testimony does not show that a person of ordinary skill in the art would not understand the boundaries of the three-step process laid out in claim 1 of the '530 patent. It simply shows that Bio-Rad can concoct a limitation that is not present in the claim, ask if the patent describes that limitation, and then get an answer in the negative. This is manufactured uncertainty — not indefiniteness.

As to Bio-Rad's continuous-process indefiniteness argument, Bio-Rad Pet. at 53–55, the argument fails because it is based on a faulty premise: that the ID's construction does not require the steps to be performed in order. *Id.* at 54. That is not the case. The ID, as well as Order Nos. 35 and 22, all require step (b) to be completed before step (c). Thus, the ID does not permit an assembly-line style process where step (c) is completed on a droplet as soon as it is generated in step (b). Bio-Rad, however, appears to mean something different when it refers to performing the steps of the claim in order. In Bio-Rad's view, no barcoding can occur in any droplet before at

least 1,000 droplets are generated in step (b). This is something more than simply requiring the steps be performed in order. What Bio-Rad seeks is to include a new negative limitation in claim 1 that excludes any barcoding from occurring before at least 1,000 droplets have been generated. This was the issue that was clarified in Order No. 35, and the basis of Bio-Rad's unsuccessful motion for summary determination of noninfringement.

Claim 1, however, is an open-ended claim, and thus other non-recited activity may occur that will not defeat infringement. Here, as 1,000 droplets are generated in step (b), there may be some barcoding happening as soon as each droplet is generated. This will not preclude the process from reading on step (c) though if, after 1,000 droplets are generated, barcodes are released in those droplets and a plurality of polynucleotides are barcoded. The fact that barcoding of other polynucleotides also happened before 1,000 droplets were generated is irrelevant. Bio-Rad incorrectly characterizes the ALJ's observation to that effect as permitting a continuous process. The ALJ correctly determined that extraneous unrecited activity will not defeat infringement of a claim drafted in open language.

Finally, we note that Bio-Rad offers no real reasoning why construing claim 1 to encompass a continuous process would render it indefinite. Bio-Rad simply parrots the reasonable certainty language of *Nautilus*. Bio-Rad Pet. at 54–55.

For all these reasons, the Commission finds that Bio-Rad waived the indefiniteness arguments raised in its petition for review, but even if not waived, those arguments and the evidence presented therein would fail to establish that claim 1 is indefinite by clear and convincing evidence.

VII. INVENTORSHIP

The Commission determined to review the ID's findings with respect to Bio-Rad's inventorship defense. *See* Notice at 2. On review, the Commission has determined to take no

position on whether Dr. Heredia should have been named as a joint inventor of the '204 patent. The Commission affirms the ID's findings with respect to Bio-Rad's inventorship defense for the other three patents. Because the Commission has affirmed the ID's finding of noninfringement with respect to the '204 patent, the Commission's determination to take no position on Bio-Rad's inventorship defense with respect to the '204 patent does not affect the ID's ultimate finding of no violation with respect to the '204 patent.

VIII. OWNERSHIP

The ID rejected Bio-Rad's claim that it had an ownership interest in each of the asserted patents based on work done by Drs. Hindson and Saxonov during their time at QuantaLife/Bio-Rad. *See* ID at 136–152. The ID began by explaining that inventorship and ownership are distinct issues, and that while federal patent law governs inventorship, ownership is a question of state contract law. *Id.* at 136–141. The ID noted with disapproval that the parties conflated the two issues in their briefing. *See id.* at 141. The ID went on to explain that the crux of the dispute with respect to Bio-Rad's ownership defense involves defining the "inventive concept" in the asserted patents. *See id.* The ID rejected Bio-Rad's approach to that issue, explaining that Bio-Rad "briefed the matter as if it owned a share of the patents because it could trace some elements of the asserted patents to work done at Quanta/Life and Bio-Rad." *Id.* The ID explained that while Bio-Rad "owns many ideas conceived by Drs. Hindson and Saxonov, [] it does not own the idea for the specific arrangement of elements claimed in the asserted patents . . . because there is insufficient evidence that that idea was conceived-during the period of employment." *Id.* at 142.

Concerning the pertinent contract language, the ID noted that "[n]o provision of any of the applicable contracts governs future inventions that are based on or developed from work done during employment." *Id.* at 144. Based on this observation, the ID found Bio-Rad's interpretation of the contract to be unreasonable because it "read out the plain meaning of the durational

limitation in the pertinent contracts, and in its place suggest[ed] an interpretation of the contracts in which inventions developed by the employee after his employment belong to the company if they are related to ideas conceived during employment." *Id.* at 145. The ID went on to reject Bio-Rad's theory that it is entitled to a pro-rata undivided co-ownership interest in the asserted patents based on Drs. Hindson and Saxonov's discovery of ideas that are related to the invention in the asserted patents, as opposed to their actual discovery of the invention. *See id.*

The ID next considered whether Bio-Rad had presented evidence showing that the inventive idea embodied in the asserted patents was conceived at QuantaLife/Bio-Rad. The ID concluded that Bio-Rad presented no direct evidence of such conception. *See id.* As for circumstantial evidence, the ID determined that the relatively short time between when Drs. Hindson and Saxonov left Bio-Rad and when they filed their first provisional patent application did not, on its own, establish conception by Drs. Hindson and Saxonov at Bio-Rad. *Id.* at 146.[14] The ID also rejected several challenges to Dr. Hindson's credibility. *Id.* at 147–48.

Next, the ID rejected Bio-Rad's argument that certain concepts disclosed by Drs. Hindson and Saxonov at Bio-Rad can be traced to the asserted patents such that conception at Bio-Rad should be implied. *Id.* at 149. In rejecting this argument, the ID credited testimony from Dr. Saxonov that the ideas formed at Bio-Rad were only directions for further research, as opposed to ideas that would work. *See id.* at 149–150. The ID also rejected a similar argument based on the '059 patent's disclosure of certain numerical ranges, *see id.* at 150, and based on lab notebooks offered by 10X. *See id.* at 150–51. The ID concluded as follows: "In sum, the evidence before me is insufficient to permit the conclusion that, more likely than not, the work Drs. Hindson and

[14] The ID noted that Drs. Hindson and Saxonov left Bio-Rad in April 2012 and founded 10X several months later. ID at 146. In August 2012, Drs. Hindson and Saxonov filed their first provisional patent application at 10X. *Id.*

Saxonov did at QuantaLife and Bio-Rad led them to conceive the idea described in the 10X patents while they were still under contract." *Id.* at 151. Accordingly, the ID found that Bio-Rad "failed to establish ownership of the asserted patents." *Id.*

The ownership dispute in this investigation revolves around Drs. Hindson and Saxonov's employment contracts with QuantaLife and Bio-Rad. The relevant portions of the QuantaLife contracts contain identical language, as follows:

RX-0623C (Hindson-QuantaLife contract) at ¶ 2; RX-0624C (Saxonov-QuantaLife contract) at ¶ 2; *see also* ID at 143–44 (quoting same). The relevant portions of the Bio-Rad contracts also contain identical language, as follows:

[redacted]

RX-0619C (Hindson-Bio-Rad employment agreement) at ¶¶ 3, 6; RX-0620C (Saxonov-Bio-Rad employment agreement) at ¶¶ 3, 6; *see also* ID at 144 (quoting same).

The Commission finds that Bio-Rad has failed to show that the "ideas" developed by Drs. Hindson and Saxonov at QuantaLife/Bio-Rad would entitle them to an ownership interest in the asserted patents. This follows for several reasons. First, in its response to the Commission's questions, Bio-Rad only attempted to map the ideas developed at QuantaLife/Bio-Rad onto a single claim: claim 1 of the '468 patent. *See* Bio-Rad Resp. to Qs. at 4–12. Bio-Rad summarily asserted that the "'468 Patent is representative of the claims of the four 10X Patents," *id.* at 5, but did not attempt to show a direct correspondence between the "ideas" developed at QuantaLife/Bio-Rad and the particular limitations of any claim of the '024, '204, and '530 patents.[15] Instead, Bio-Rad argued that all four asserted patents have the same "fundamental architecture," and thus its mapping of ideas onto the limitations of claim 1 of the '468 patent should entitle it to an ownership interest in the other asserted patents as well. *See id.* at 12–14. Thus, at best, Bio-Rad's showing of ownership under its theory would be limited to the '468 patent.

Second, Bio-Rad was only able to map the "ideas" it relies on to claim 1 of the '468 patent because it substituted generic descriptions in place of the specific limitations of that claim. For example, Bio-Rad argued that Dr. Hindson "came up with ideas at QuantaLife about [redacted]

[15] Among other "ideas," Bio-Rad argued that Drs. Hindson and Saxonov conceived of the idea to use porous gel beads as a reagent delivery system while at QuantaLife. *See* Bio-Rad Resp. to Qs. at 10. However, Order No. 43 precluded Bio-Rad from arguing that the idea for porous gel beads was conceived at QuantaLife/Bio-Rad. Bio-Rad did not petition for review of that order, nor has the Commission determined to review that order *sua sponte*. Accordingly, Bio-Rad may not now argue that it is entitled to an ownership interest in the asserted patents because the idea of using porous gel beads was developed at QuantaLife.

[redacted] and that "[t]he use of droplets to partition sample (and achieve a single cell per partition) is fundamental to claim 1 of the '468 Patent." Bio-Rad Resp. to Qs. at 5. But claim 1 of the '468 patent recites a method for droplet generation with three steps, each of which has a number of specific internal limitations; it does not broadly claim the use of droplets to partition a sample. *See* '468 patent at cl. 1. That disconnect undercuts Bio-Rad's theory of ownership based on Drs. Hindson and Saxonov's prior "ideas."

In the same vein, the Commission also notes that the "ideas" Bio-Rad identified relate to different architectures and applications than those central to the asserted patents. *See* CX-0001C (Hindson WS) at Q/A 79–107 (discussing 10X's development of its GEMs and their attributes); *see also* ID at 142 ("the inventive idea is a specific arrangement of elements which, when combined, works to achieve a desired goal."). This follows from the fact that the "ideas" relied on by Bio-Rad were developed in connection with the droplet-in-droplet architecture described in the '059 patent. *See, e.g.*, Bio-Rad Pet. at 84, 87 (citing lab notebook (RX-127C at 95, 97) and [redacted], to support ownership claim based on "ideas" developed at QuantaLife). The asserted patents, however, do not use a droplet-in-droplet approach, as the '059 patent did (Dr. Saxonov is the named inventor of the '059 patent, and he assigned the patent to Bio-Rad). *See* Tr. (Metzker) at 656–657; CX-1829C (Saxonov WS) at Q/A 28–32 (discussing the droplet-in-droplet concept for barcoding before sequencing and its disclosure in the '059 patent); CX-1827C (Dear WS) at Q/A 40. Rather, the asserted patents, in contrast, require features such as the release of the barcodes from the bead into the droplet in the '024 patent, a particular microfluidic arrangement for generating droplets with the beads in the '468 patent, and a large diversity of beads for use in

generating droplets with single cells in the '530 patent. *See* CX-1827C (Dear WS) at Q/A 40; *see also* ID at 33–40 (finding that the '024 patent was novel and not obvious vis-à-vis the '059 patent and Church (RX-0462)). As such, the asserted patents are based on a different architecture involving beads or capsules that release key reactants. *See* CX-1828C (Hindson WS) at Q/A 24–34 (describing how 10X invented its GEM architecture "from scratch . . . because there was no such architecture at QuantaLife."). Thus, the inventions claimed in the asserted patents are fundamentally different from the prior work conducted at QuantaLife/Bio-Rad.

Third, even under Bio-Rad's theory that it owns a share of the patents based on joint inventorship principles, *see, e.g.*, Bio-Rad Pet. at 77–80, Bio-Rad has not shown that the "ideas" it relies on to build its joint inventorship argument are distinct from the prior art. Indeed, many of these "ideas" are embodied in the '059 patent — a patent naming Dr. Saxonov as an inventor that *was* assigned to Bio-Rad because the underlying invention was developed during his employment at Bio-Rad — which make those ideas part of the prior art. *See* '059 patent (JX-0031) at 1:26–55. But merely explaining the prior art is not sufficient to render someone a joint inventor. *See Fina Oil & Chem. Co. v. Ewen*, 123 F.3d 1466, 1473 (Fed. Cir. 1997) ("[A] person will not be a co-inventor if he or she does no more than explain to the real inventors concepts that are well known and the current state of the art."). No part of Drs. Hindson and Saxonov's employment agreements preclude them from building on ideas in the prior art. Moreover, the existence of the '059 patent demonstrates that Bio-Rad received the benefit of its bargain with respect to the employment agreements. For the ideas that were conceived at QuantaLife or Bio-Rad, Dr. Saxonov did assign his rights. *See* '059 patent (JX-0031) at Cover ("Assignee: Bio-Rad Laboratories, Inc."). Bio-Rad overreaches insomuch as it now attempts to extend its rights to inventions conceived outside the term of Drs. Hindson and Saxonov's employment agreements. *Cf. Israel Bio-Eng'g Project v.*

Amgen, Inc., 475 F.3d 1256, 1267 (Fed. Cir. 2007) (in a case involving an Israeli contract, the Federal Circuit concluded that the plaintiff "was not entitled to further assignments of any other newly developed inventions, even when these inventions built on proprietary information developed during the [contractual] R & D process," which concluded in December 1987); *see also* ID at 148–49 n.29 (reasoning that if Hindson and Saxonov's prior, generic work [redacted] were sufficient to trigger ownership rights, "the contracts' [redacted] would be nullities."); *Dawson v. Dawson*, 710 F.3d 1347, 1353–56 (Fed. Cir. 2013) (concluding that, along with other evidence, a preliminary statement about a potential use was insufficient to establish that an inventor conceived the claimed invention while employed by his former employer). Accordingly, for the reasons provided above, the Commission finds that Bio-Rad has failed to show that the "ideas" Bio-Rad relies on entitle it to an ownership interest in the asserted patent.

Concerning the ID's use of the phrase "inventive concept," the Commission notes that the phrase has some history in patent law and its use in the ID may invite confusion, as evidenced by Bio-Rad's brief. *See, e.g.,* Bio-Rad Ans. at 16 ("The ALJ's analysis was incorrect because it treated the ownership question as requiring proof of a singular eureka moment at a specific point in time when everything was finalized and established to work."). Particularly, "inventive concept" may imply similarity to the pre-1952 patent law's requirement for a "flash of genius," *compare Cuno Eng'g Corp. v. Automatic Devices Corp.*, 314 U.S. 84, 91 (1941) (requiring an invention to "reveal the flash of creative genius not merely the skill of the calling.") *with* Pub. L. 82-593, § 103, July 19, 1952, 66 Stat. 798 (Patent Act of 1952) ("Patentability shall not be negatived by the manner in which the invention was made."), or it may suggest the search for an "inventive concept" in step 2 of an *Alice* patent-eligibility analysis. *See Alice Corp. Pty. v. CLS*

Bank Int'l, 573 U.S. 208, 217 (2014) ("We have described step two of this analysis as a search for an 'inventive concept.'").

Upon review of the ID, the Commission has determined to clarify that the ID's use of the phrase "inventive concept" is synonymous with "the specific arrangement of elements claimed in the asserted patents." ID at 142; *see also id.* ("[T]he invention claimed in the asserted patents is complex and consists of many elements. CX-0001C (Hindson WS) at Q/A 88. The inventive idea, which emerged from many other ideas (some of which clearly were in the prior art), is to combine these elements in a process resulting in what 10X calls the GEM ('gel bead in emulsion') architecture. As confirmed by both parties, **the inventive idea is a specific arrangement of elements which, when combined, works to achieve a desired goal**."). Bio-Rad's position that the use of the phrase "inventive concept" in the ID is indicative of a search for a singular eureka moment conflicts with the ID's explanation that the inventive concept is the combination and specific arrangement of elements laid out in the claims of the asserted patents. The Commission finds no error in the ID's focus on the inventions as laid out in the claims in its analysis of Bio-Rad's ownership defense.

Consistent with the reasoning above, the Commission affirms with supplemented reasoning the ID's finding that Bio-Rad has not shown that it is entitled to an ownership interest in any of the asserted patents.

IX. CLERICAL ERROR

10X's petition for review included a request to correct two clerical errors in the ID. *See* 10X Pet. at 18–19. One of the errors appears on page 91 of the ID, and the other on page 105. *See id.* at 19. The error on page 105 relates to the same absence of an accused assay in the ID's infringement findings for dependent claim 26 of the '530, which has already been addressed *supra* in this opinion. Concerning the error on page 91, 10X explained that "[t]he ID states on page 91

that '[i]n Order No. 35, this claim construction was further clarified so that it does preclude the generation of some barcoded molecules before the start of the claimed third step,' which should have stated 'so that it does *not* preclude the generation of some barcoded molecules before the start of the claimed third step.'" *Id.* OUII agreed that the omission of the word "not" was an oversight. *See* OUII Resp. to Pets. at 44–45. Bio-Rad did not directly respond to 10X's assertion that the omission of the word "not" was a clerical error. *See generally* Bio-Rad Resp. to Pets. Instead, through its own petition, Bio-Rad pointed to the absence of the word "not" as evidence of "contradictory statements" by the ALJ for the purpose of bolstering its argument that the ALJ adopted two contradictory claim constructions for the '530 patent in Order No. 22 and Order No. 35. *See* Bio-Rad Pet. at 46, n.7.

Upon review of Order No. 35, the Commission agrees with 10X and OUII that the omission of the word "not" on page 91 of the ID is a simple clerical error. *Cf.* Order No. 35 ("Bio-Rad reads the claims to require 'that all 1,000 droplets form before any barcoding begins,' Reply at 8, but no such limitation was contemplated in the *Markman* order. The claim language merely requires that any accused step of generating a plurality of barcoded molecules occurs after the at least 1,000 droplets are generated."). Bio-Rad's attempt to frame that error as evidence of contradictory statements by the ALJ is not persuasive. Accordingly, the last sentence of the first full paragraph on page 91 of the ID is modified to read: "In Order No. 35, this claim construction was further clarified so that it does *not* preclude the generation of some barcoded molecules before the start of the claimed third step."

X. REMEDY

The RD recommended that the Commission issue an LEO and CDO directed to Bio-Rad. There was no dispute among the parties that an LEO would be the appropriate remedy. *See* RD at 1. The RD also explained that while Bio-Rad "suggest[ed]" that the LEO should include a

certification provision, "there is no evidence in the record that a certification provision will be necessary to distinguish between infringing and non-infringing products," and on that basis declined to recommend the inclusion of a certification provision. *Id.* at 2.

With respect to the CDO, the RD found that Bio-Rad maintains a commercially significant domestic inventory of ddSEQ products and on that basis recommended that the Commission issue a CDO directed to Bio-Rad.[16] *See id.* at 2–3. Specifically, the RD found that Bio-Rad had inventory of ddSEQ Single-Cell Isolators and ddSEQ-M cartridges in California. *See id.* at 2. The RD found these inventories to be significant because the number of units in inventory exceeded the number of such units Bio-Rad actually sold between 2017 and 2018. *See id.* While there was a dispute regarding whether some number of the cartridges should be discounted because they were for testing purposes, the RD agreed with 10X's expert, Dr. Vander Veen, that the inventory of cartridges would be significant even if the test cartridges were not considered. *See id.* at 2–3.

A. Limited Exclusion Order

Section 337(d)(1) provides that "[i]f the Commission determines, as a result of an investigation under this section, that there is a violation of this section, it shall direct that the articles concerned, imported by any person violating the provision of this section, be excluded from entry into the United States, unless, after considering the [public interest], it finds that such articles should not be excluded from entry." 19 U.S.C. § 1337(d)(1). The Commission has "broad discretion in selecting the form, scope, and extent of the remedy." *Viscofan, S.A. v. US. Int'l*

[16] As explained in *Certain Road Construction Machines and Components Thereof*, "[t]he Commission generally issues cease and desist orders with respect to the imported infringing products when 'respondents maintain commercially significant inventories in the United States or have significant domestic operations that could undercut the remedy provided by an exclusion order.'" Inv. No. 337-TA-1088, Comm'n Op. at 51 (June 27, 2019) (quoting *Certain Table Saws Incorporating Active Injury Mitigation Technology and Components Thereof*, Inv. No. 337-TA-965, Comm'n Op. at 4 (Jan. 27, 2017)).

Trade Comm'n, 787 F.2d 544, 548 (Fed. Cir. 1986). Thus, the Commission may issue an LEO excluding the goods of the person(s) found in violation.

Here, all parties agree that an LEO is appropriate in this investigation should the Commission affirm the ID's finding of a violation, and we agree that an LEO is appropriate here. There are, however, questions about the scope of that LEO and the exemptions it should contain. The questions concern: (1) whether the LEO should include an exemption for all ddSEQ v2 products ("v2 product exemption"); (2) whether the LEO should include exemptions for any product used for warranty, repair, or service purposes, and/or for consumables for existing deployments of Bio-Rad's ddSEQ v1 products ("existing use exemptions"); (3) whether the LEO should include an exemption for internal research and development testing by Bio-Rad ("internal research and development exemption"); and (4) whether a certification of noninfringement provision should be included in the LEO ("certification provision").[17] The parties disagree on questions (1), (3) and (4) but agree that the LEO should include existing use exemptions.

1. v2 Product Exemption

The most significant disagreement between the parties is whether the LEO should explicitly exempt the ddSEQ v2 products because the ID found that 10X did not establish indirect infringement of those products. Bio-Rad seeks an exemption for its ddSEQ v2 products on the

[17] 10X also includes a section explaining that Bio-Rad has admitted "that the scATAC-seq assay is now commercially available and has been used by its customers in the United States," and therefore "Bio-Rad now also contributorily infringes 10X's Asserted Patents through sales of the scATAC-seq assay and induces infringement of others' uses of its scATAC-seq assay." 10X Resp. to Qs. at 55–56. The purpose of 10X's briefing on this point is far from clear, but it appears that 10X is asking the Commission to expand the indirect infringement findings in the ID to include the scATAC-seq assay, though it fails to explicitly make that request. To the extent 10X intends to request a Commission ruling as to whether the scATAC-seq assay indirectly infringes, the Commission's Rules provide procedures for obtaining such as ruling through a request for an advisory opinion or a petition for modification of the remedial orders. *See* 19 C.F.R §§ 210.76, 210.79.

basis that the ID found no indirect infringement due to the fact that the products were not available for commercial sale and had not yet been used in the United States, which necessarily precluded a finding of indirect infringement due to an underlying lack of direct infringement. *See* Bio-Rad Resp. to Qs. at 72–73. 10X counters that the ID nonetheless found the v2 products to be infringing, just like the v1 products, and that the Commission's longstanding practice has been to direct its exclusion orders broadly to articles that infringe, whether those articles currently exist or if they are manufactured and imported in the future. *See* 10X Reply at 58–59. OUII's position is that the v2 products should not be exempted because the ID did not foreclose the possibility that the importation of the v2 products would constitute a violation of section 337 if the requirements for indirect infringement are later met. *See* OUII Reply at 22. OUII does, however, recommend including a certification provision in the LEO allowing Bio-Rad to certify that either the v1 or v2 products are imported for use in a noninfringing manner. *See id.* at 22–23.

The ID uses a two-step approach to its infringement analysis. First, for each asserted patent, the ID determines whether the accused products practice the limitations of the asserted claims of that patent. Those determinations revolve around an analysis of how the microfluidic chips and instruments operate when used with the assays specific to those chips, *i.e.*, the v1 chips with the WTA 3' v1 assay, and the v2 chips with the [redacted], scATAC-seq,[18] [redacted] *See* ID at 3 (listing assays for the v1 and v2 ddSEQ systems). For the '024 and '468 patents, the ID found that the v1 and v2 systems/processes infringe all of the claims asserted from those patents. *See id.* at 27, 62–63. For the '530 patent, only the WTA 3' v1 [redacted] scATAC-seq, and [redacted] assays were accused. *See id.* at 91. The ID found that all of those

[18] The ID also includes a finding that shows that the scATAC-seq assay can be used with a v1 cartridge. *See* ID at 96 ("If the scATAC-seq assay is performed using the ddSEQ vl cartridge, each lane is capable of generating 500 droplets with a cell and gel bead.").

accused products infringe independent claim 1 of the '530 patent. *See id.* at 102–103. For the dependent claims of the '530 patent, the ID found infringement with respect to all of the asserted dependent claims and all of the accused products except in two instances. The ID explicitly found that the scATAC-seq assay does not infringe claim 4, and the ID omitted [redacted] from the list of assays that infringe claim 26. *See id.* at 103, 104. As explained above, the omission of the [redacted] assay from the claim 26 findings is an inadvertent error that the Commission has corrected on review. Accordingly, for the '530 patent, there is a single accused assay — scATAC-seq — that does not infringe one particular asserted dependent claim: dependent claim 4.

The second step in the ID's analysis was the determination of whether Bio-Rad induced or contributed to the infringement of any of the asserted claims. Of particular importance here, for each of the '024, '468, and '530 patents, the ID first considered whether there was an underlying act of direct infringement that could support a finding of indirect infringement. For each of the '024, '468, and '530 patents, the ID found that an act of direct infringement had occurred with respect to the v1 products but not the v2 products. The failure as to the v2 products was based on the fact that 10X could not show actual use of the v2 products in the United States by entities other than Bio-Rad at the time of the hearing. *See* ID at 28–29, 64, 105–108. Because the ID found no act of direct infringement with respect to the v2 products, it did not make findings about whether Bio-Rad induced infringement with the v2 products, or if the v2 products have a substantial noninfringing use.

Upon review of the parties' submissions, the Commission has determined not to adopt an exemption for the v2 products. The Commission's established practice is to direct its remedial orders to articles that infringe, as opposed to specific product model numbers. *See Certain Hardware Logic Emulation Systems and Components Thereof*, Inv. No. 337-TA-383, USTIC Pub.

3089 (Mar. 1998), Comm'n Op. on Remedy, the Public Interest, and Bonding at 16 ("The limited exclusion order is not limited to the specific models of emulation system found by the Commission to infringe, as urged by respondents. As the ALJ noted, the Commission's long-standing practice is to direct its remedial orders to all products covered by the patent claims as to which a violation has been found, rather than limiting its orders to only those specific models selected for the infringement analysis. As the IAs noted, while individual models may be evaluated to determine importation and infringement, the Commission's jurisdiction extends to all models of infringing products that are imported at the time of the Commission's determination and to all such products that will be imported during the life of the remedial orders.").

2. Existing Use Exemptions

There is broad agreement among the parties that certain exemptions to the LEO *are* appropriate. These consist of an exemption for customers who currently have access to ddSEQ equipment to continue to purchase repair parts and warranty replacements as well as consumables. *See* 10X Resp. to Qs. at 59–60; Bio-Rad Resp. to Qs. at 73–74; OUII Reply at 23. These exemptions will allow the work of researchers already using Bio-Rad's products to continue. Consistent with the existing use exemption adopted in the LEO and CDO issued in *Certain Microfluidic Devices*, Inv. No. 337-TA-1068 ("the 1068 investigation"),[19] researchers seeking to

[19] In the 1068 investigation, Bio-Rad was the complainant and 10X was the respondent. *See* 82 Fed. Reg. 42115 (Sep. 6, 2017). The Commission found that 10X had violated section 337 through the importation of microfluidic devices that infringed Bio-Rad's patents. *Certain Microfluidic Devices*, Inv. No. 337-TA-1068, Comm'n Op. at 1 (Jan. 10, 2020) (public version). Due to substantial public interest concerns and supporting record evidence, particularly with respect to the public health and welfare, the Commission tailored its remedial orders in the 1068 investigation to exempt otherwise covered microfluidic devices, provided that scientists and medical researchers using those devices established that they had a documented need to continue receiving the devices to continue ongoing research and that no alternative product could be substituted for the covered microfluidic device. *See id.* at 46.

receive ddSEQ consumables under that provision must provide Bio-Rad with a documented need to continue receiving those consumables for an identified current ongoing research project for which that need cannot be met by any alternative product. With respect to warranty and repair parts, the orders also exempt service or repair articles imported for use in servicing or repairing microfluidic systems that were imported as of the date of this Order and are under a warranty that existed as of the date of this Order, if such servicing or repairing is provided for in terms of the warranty.

The Commission's remedial orders include as attachments questionnaires that Bio-Rad is to provide to its customers for purposes of obtaining infringing ddSEQ consumables after the effective date of the Commission's orders. Bio-Rad may provide a modified version of that questionnaire to its customers, but whatever documentation it uses must request from its customers at least the information requested in the attached questionnaires using the verbiage as it appears in the questionnaires. A completed questionnaire (or its modified equivalent) establishes a "documented need" to qualify for the exemption, as that phrase is used in this opinion. The questionnaires request, *inter alia*, a researcher to identify the date the research for which he or she is using the ddSEQ system began and to state whether other products could meet his or her research needs. The questionnaires also require both Bio-Rad and its customers to certify their statements and to acknowledge that U.S. law (including, but not limited to, 18 U.S.C. § 1001) imposes criminal sanctions on individuals who knowingly and willfully make material false statements to the U.S. Government. To qualify for the exemption, the researcher must attest in the questionnaire that the research using the ddSEQ system began prior to the date of issuance of these remedial orders, and also attest that other products cannot meet his or her research needs. In addition, researchers who avail themselves of this exemption are required to maintain records to support

their declarations in case an audit is carried out or such records are required for any future enforcement proceeding. These accompanying records are not to be provided to Bio-Rad.

United States Customs and Border Protection ("CBP") may choose to require Bio-Rad to furnish the relevant completed questionnaires for each entry that is claimed to be exempted. *See* LEO, at ¶¶ 2–3. CBP may require that the questionnaires be submitted in advance of the date of entry of the ddSEQ consumables and pursuant to procedures that CBP establishes. The recordkeeping provision of the CDO requires Bio-Rad to retain such questionnaires, and the reporting provision requires Bio-Rad to report such records. *See* CDO, at §§ V, VI.

Consistent with the 1068 investigation, the CDO in this investigation requires Bio-Rad to provide a detailed accounting showing that the consumables imported and/or sold in the United States after importation (including sales of any infringing domestic inventory existing at the time of the Commission's decision) are being sent to only those identified customers and that consumables are not being stockpiled, sent to unauthorized customers, or used for research projects other than those identified. *See* CDO at § V. That accounting must be supported by documentation (including the questionnaires) referencing all relevant information, including the number of consumables imported and/or sold and the identity of the customers, their exempted research project(s), and the projected completion date of such projects. The reporting provision requires monthly, rather than the Commission's standard annual, reports.

3. Internal Research and Development Exemption

Bio-Rad also seeks an exemption for its internal research and development testing by Bio-Rad; 10X has not acquiesced to that exemption. *See* Bio-Rad Resp. to Qs. at 74; 10X Reply at 57. Bio-Rad makes two arguments in favor of such an exemption. The first is that the Commission has incorporated such exemptions before. *Id.* (citing *Certain Devices for Connecting Computers*

via Tel. Lines, Inv. No. 337-TA-360, Comm'n Op. at 7–10 (Nov. 18, 1994) ("A complainant that seeks exclusion of other types of entry [other than for consumption] should present evidence that activities by respondents involving other types of entry either are adversely affecting it or are likely to do so."); *Certain Magnetic Data Storage Tapes and Cartridges Containing the Same*, Inv. No. 337-TA-1012, Comm'n Op. at 128–133 (Apr. 2, 2018) ("*Magnetic Storage Tapes*") (exempting infringing products used for U.S.-based compliance testing that was necessary for foreign sales)). The second argument is that because the asserted claims for which a violation was found are method claims, Bio-Rad's own use of its products cannot be a violation of Section 337. *See* Bio-Rad Reply at 55 (citing *Electronic Devices with Image Processing Systems, Components Thereof, and Associated Software*, Inv. No. 337-TA-724, Comm'n Op at 18-20 (Dec. 1, 2011)). 10X opposes this exemption on the basis that Bio-Rad waived it by failing to ask for it in briefing before the ALJ, and that the cases relied on by Bio-Rad are factually distinguishable from this investigation. *See* 10X Reply at 57–58. OUII also opposes an exemption for internal development and testing purposes. *See* OUII Reply at 23.

The Commission has determined not to include an exemption for internal development and testing. Neither of the cases Bio-Rad cited in its initial response to the Commission's questions stand for the proposition that an "entry for consumption" excludes research and development uses. Further, Bio-Rad has not established an evidentiary basis to support a need for this exemption in contrast to the respondent in *Magnetic Storage Tapes.* *See* Comm'n Op. at 132 (finding that denial of an exemption for compliance verification testing would amount to a "world-wide" prohibition against Sony's products, since verification testing in the United States appears to be necessary even for foreign sales of Sony's LTO-7 products). Bio-Rad's request that it be allowed to continue importing infringing products for research and development purposes finds no precedent as a matter of patent law or section 337. As the Federal Circuit has recognized, there "is no fair use or

research and development exception for infringement of normal commercial processes." *Soitec, S.A. v. Silicon Genesis Corp.*, 81 F. App'x 734, 737 (Fed. Cir. 2003) (citing *Madey v. Duke Univ.*, 307 F.3d 1351, 1362 (Fed. Cir. 2002) (stating that "the experimental use defense is . . . limited to actions performed 'for amusement, to satisfy idle curiosity, or for strictly philosophical inquiry.'") (citation omitted)). Likewise, Bio-Rad points to no Commission investigation where a respondent was allowed to continue importing its own products, which had been found in violation, for such internal testing purposes that would continue to infringe the patents.

4. Certification Provision

Finally, the parties dispute whether a certification of noninfringement provision should be included with the exclusion order. 10X argues that no certification provision is appropriate because here, unlike in the 1068 investigation, there is no evidence that the determination of whether a Bio-Rad product is infringing will be technically difficult. *See* 10X Resp. to Qs at 57–58. OUII supports including a certification provision "because it is possible that certain accused 'v2 products' will not infringe if imported, and because it is possible that the accused products could be used in non-infringing ways." OUII Resp. to Qs at 28. Bio-Rad joins OUII's reasoning and also argues that a certification provision will facilitate enforcing the exemptions on which the parties agree. Bio-Rad Reply at 56.

Upon consideration of the parties' submissions, the Commission has determined to include a standard certification provision in the LEO to facilitate CBP's enforcement of the order. *See Certain Composite Aerogel Insulation Materials and Methods for Manufacturing the Same*, Inv. No. 337-TA-1003, Comm'n Op. at 62 (Feb. 22, 2018) ("[T]he Commission's standard practice for the past several years [has been] to include certification provisions in exclusion orders to aid CBP."). This provision does not, however, provide Bio-Rad with the ability to self-certify that its

products are noninfringing. That determination must be made by the Commission or CBP. *See id.* ("CBP only accepts a certification that the goods have been previously determined by CBP or the Commission not to violate the exclusion order."). The standard certification can be used to facilitate entry of products adjudicated to be non-infringing as well as for products imported for warranty and repair service pursuant to the express terms of Bio-Rad's warranty provisions. In addition to the standard provision, the LEO provides a separate procedure by which Bio-Rad may certify that the microfluidic devices are being imported for use by researchers who have been using such devices in the United States as of the date of the issuance of the LEO, and who have provided Bio-Rad a documented need to continue receiving the devices for an identified current ongoing research project for which that need cannot be met by any alternative product.

B. Cease and Desist Order

Section 337(f)(1) provides that in addition to, or in lieu of, the issuance of an exclusion order, the Commission may issue a CDO as a remedy for violation of section 337. *See* 19 U.S.C. § 1337(f)(1). CDOs are generally issued when, with respect to the imported infringing products, respondents maintain commercially significant inventories in the United States or have significant domestic operations that could undercut the remedy provided by an exclusion order.[20] *See, e.g.*, *Certain Table Saws Incorporating Active Injury Mitigation Technology & Components Thereof* ("*Table Saws*"), Inv. No. 337-TA-965, Comm'n Op. at 4-6 (Feb. 1, 2017); *Certain Protective Cases & Components Thereof*, Inv. No. 337-TA-780, USITC Pub. No. 4405, Comm'n Op. at 28

[20] When the presence of infringing domestic inventory or domestic operations is asserted as the basis for a CDO under section 337(f)(1), Commissioner Schmidtlein does not adopt the view that the inventory or domestic operations needs to be "commercially significant" in order to issue the CDO. *See, e.g.*, *Certain Magnetic Tape Cartridges and Components Thereof*, Inv. No. 337-TA-1058, Comm'n Op. at 65, n.24 (Mar. 25, 2019); *Table Saws*, Comm'n Op. at at 6-7, n.2 (Feb. 1, 2017). In Commissioner Schmidtlein's view, the presence of some infringing domestic inventory or domestic operations, regardless of its commercial significance, provides a basis to issue a CDO. *Id.*

(Nov. 19, 2012) (citing *Certain Laser Bar Code Scanners & Scan Engines, Components Thereof & Prods. Containing Same*, Inv. No. 337-TA-551, Comm'n Op. at 22 (June 24, 2007)). Complainants bear the burden on this issue. "A complainant seeking a cease and desist order must demonstrate, based on the record, that this remedy is necessary to address the violation found in the investigation so as to not undercut the relief provided by the exclusion order." *Table Saws*, Comm'n Op. at 5 (citing *Certain Integrated Repeaters, Switches, Transceivers, & Prods. Containing Same*, Inv. No. 337-TA-435, USITC Pub. No. 3547 (Oct. 2002), Comm'n Op. at 27 (Aug. 16, 2002); *see also* H.R. REP. No. 100-40, at 160 (1987)).

The RD recommended issuing a cease and desist order based on its finding that Bio-Rad maintains a commercially significant inventory of ddSEQ products in the United States. RD at 2–3. Both 10X and OUII supported the RD's recommendation. *See* 10X Resp. to Qs. at 58–59; OUII Resp. to Qs. at 29. Bio-Rad opposed the recommendation and argued that 10X's expert incorrectly included noninfringing test chips in his analysis of Bio-Rad's inventory. *See* Bio-Rad Reply at 56–57.

The Commission has determined to adopt the RD's recommendation and issue a cease and desist order to Bio-Rad. The RD considered the argument Bio-Rad raised, and determined that even if the test chips were discounted, the inventory of ddSEQ chips in the United States would still be commercially significant. RD at 2–3 ("I agree with 10X and Dr. Vander Veen that regardless of whether the 'test' cartridges are counted, Bio-Rad's inventory of ddSEQ products is commercially significant."). Bio-Rad has shown no error in that finding, which is supported by record evidence. *See* CX-0005C at Q/A 39.

Like the LEO discussed above, the CDO exempts from its scope the importation of certain microfluidic consumables for use by researchers who have been using such consumables in the

United States as of the date of the issuance of the CDO, and who have provided Bio-Rad a documented need to continue receiving the consumables for an identified current ongoing research project for which that need cannot be met by any alternative product. The CDO also exempts from its scope service or repair articles imported for use in servicing or repairing microfluidic systems that were imported as of the date of the issuance of the CDO and are under a warranty that existed as of the date of this Order, if such servicing or repairing is provided for in terms of the warranty

XI. BOND

If the Commission enters an exclusion order or a cease and desist order, a respondent may continue to import and sell its products during the 60-day period of Presidential review under a bond in an amount determined by the Commission to be "sufficient to protect the complainant from any injury." 19 U.S.C. § 1337(j)(3); *see also* 19 C.F.R. § 210.50(a)(3). When reliable price information is available in the record, the Commission has often set the bond in an amount that would eliminate the price differential between the domestic product and the imported, infringing product. *See Certain Microsphere Adhesives, Processes for Making Same, & Prods. Containing Same, Including Self-stick Repositionable Notes*, Inv. No. 337-TA-366, USITC Pub. No. 2949, Comm'n Op. at 24 (Jan. 16, 1996). The Commission also has used a reasonable royalty rate to set the bond amount where a reasonable royalty rate could be ascertained from the evidence in the record. *See, e.g.*, *Certain Audio Digital-to-Analog Converters & Prods. Containing Same*, Inv. No. 337-TA-499, Comm'n Op. at 25 (Mar. 3, 2005). Where the record establishes that the calculation of a price differential is impractical or there is insufficient evidence in the record to determine a reasonable royalty, the Commission has imposed a 100 percent bond. *See, e.g.*, *Certain Liquid Crystal Display Modules, Prods. Containing Same, & Methods Using the Same*, Inv. No. 337-TA-634, Comm'n Op. at 6-7 (Nov. 24, 2009). The complainant, however, bears the burden of establishing the need for a bond. *Certain Rubber Antidegradants, Components Thereof*

& Prods. Containing Same, Inv. No. 337-TA-533, USITC Pub. No. 3975, Comm'n Op. at 40 (July 21, 2006).

The RD recommended that the Commission impose a bond of 25 percent of the entered value of infringing products imported by Bio-Rad during the presidential review period. In reaching that recommendation, the RD rejected an argument from Bio-Rad that 10X had failed to show that it was injured by the importation of Bio-Rad's products. *See* RD at 4. While the RD acknowledged some contrary evidence, it ultimately credited the testimony and analysis of 10X's expert, Dr. Vander Veen, that 10X was forced to lower its prices in response to Bio-Rad's presence in the market. *See id.*

On the amount of bond, the RD reached the 25 percent figure based on a comparison of the average selling prices of Bio-Rad's ddSEQ Single-Cell Isolator and 10X's Chromium Single Cell Controller, *i.e.*, the parties' single cell instruments. *See id.* at 5. That comparison was one of two offered by Bio-Rad's expert, Mr. Herrington. *See id.* at 4–5. The RD declined to compare the cost of the parties' consumables because experts on both sides agreed that such a comparison was impractical. *See id.* The RD also rejected 10X's request for a 100 percent bond rate, which was based on 10X's assertion that no reliable price comparison could be performed at all. *See id.* at 5. The RD explained that while "Mr. Herrington's comparison between the average selling prices of the parties' single cell instruments is not perfect, [] absent any other price comparison offered by 10X, the 25 percent price differential is the most reliable evidence in the record for an appropriate bond amount." *Id.*

The Commission has determined to adopt the recommendation of the RD and impose a bond in the amount of 25 percent of the entered value of the subject articles. OUII supports that approach. *See* OUII Resp. to Qs. at 30–33. 10X and Bio-Rad do not support the RD's

recommendation, but their positions merely rehash the arguments addressed in the RD, or advance unendorsed methodologies. Particularly, 10X first argues that a price differential is not possible, and therefore a 100 percent bond is appropriate. *See* 10X Resp. to Qs. at 68–71. In support of that first argument, 10X makes three points: (1) 10X argues that the parties' [redacted] undercuts any price differential's ability to protect 10X from harm; (2) 10X argues that importation of Bio-Rad's ddSEQ system may affect 10X's Chromium product line in addition to its single-cell instrument sales, and the absence of analysis on those products precludes a reliable price comparison; and (3) 10X criticizes an alternative "price per cell" calculation Bio-Rad offered but that the RD did not adopt. *Id.* at 68–70.

As to the first point, 10X fails to explain why [redacted] precludes a price differential calculation. If 10X's position is that it is entitled to a price differential based on higher sales prices for its own products, it had months of discovery and then an evidentiary hearing to produce evidence of those higher [redacted] prices. Moreover, such evidence about 10X's own sales prices, and reasoning therefore, was in 10X's control. On the second point, 10X's argument is supported only by a handful of conclusory statements from its economic expert. This testimony does not provide sufficient justification to abandon any attempt at calculating a price differential, which is what 10X has done. *See* 10X Resp. to Qs. at 69 (citing CX-0005C at Q/A 46–51). As to 10X's third point, the RD did not rely on a price per cell calculation, and the Commission has determined not to adopt such an approach. Accordingly, the Commission declines to impose a 100 percent bond on the basis that a price comparison is impractical.

10X makes a backup argument that if a price differential can be calculated based on instrument sales, then the correct calculation yields a bond of [redacted]. *See* 10X

Resp. to Qs. at 71–73. 10X reaches these percentages by taking the difference of either the average sales or lists prices of the parties' single cell instruments and then dividing that difference by the entered value of the Bio-Rad instruments, [redacted]. *See id.* at 72. 10X asserts that this calculation is supported by *Certain Reclosable Plastic Bags and Tubing*, Inv. No. 337-TA-266, USITC Pub. 2058, Comm'n Op. at 6 (Dec. 1, 1987) ("*Reclosable Plastic Bags*"). This approach appears to be endorsed by 10X's expert, Dr. Thomas Vander Veen, as well. *See* CX-0005C at Q/A 48.

10X's calculation is without support in Commission precedent. *Reclosable Plastic Bags* stated only that CBP preferred bonds to be calculated as a percentage of entered values, so the Commission issues a bond as a percentage of entered value and not as a dollar amount per product. *Id.* at Comm'n Op. at 6. The typical method for calculating a price differential is to subtract the price of the respondent's product from the price of the complainant's product, divide the difference by the price of the respondent's product, and then multiply by 100 to reach a percentage value. *See Certain Two-Handle Centerset Faucets and Eschutcheons, and Components Thereof*, Inv. No. 337-TA-422, USITC Pub. No. 3332, Comm'n Op. on Remedy, the Public Interest, and Bonding, 2000 WL 1159298, at *10 n.13 (July 2000) (stating that "[t]he amount of the bond was derived by dividing the remainder of the ***average price*** of the Moen faucet minus the ***average price*** of the infringing Foremost/Chung Cheng faucets by the ***average price*** of the Foremost/Chung Cheng faucets, and then multiplying the result by 100"). Indeed, this appears to be the method used in *Certain Protective Cases and Components Thereof,* Inv. No. 337-TA-780, USITC Pub. 4405, Initial Determination at 121–22, (July 10, 2012), upon which 10X relies in its brief. *See* 10X Resp. to Qs. at 73 n.12. Accordingly, the Commission declines to adopt 10X's proposed calculation, which departs from the Commission's established method of calculating price differentials.

With respect to Bio-Rad, it merely argued that 10X failed to establish injury warranting a bond. Particularly, pointing to its price per cell metric, it argued that [redacted], and thus no bond at all is appropriate. Bio-Rad Resp. to Qs. at 75. As noted above though, the RD declined to adopt Bio-Rad's price per cell metric, and Bio-Rad has not shown why the Commission should adopt it. *See* RD at 5.

For the reasons provided above, the Commission has determined to impose a bond of twenty-five percent (25%) of entered value of infringing articles imported during the period of Presidential review.

XII. PUBLIC INTEREST

Section 337 requires the Commission, upon finding a violation of section 337, to issue an LEO "unless, after considering the effect of such exclusion upon the public health and welfare, competitive conditions in the United States economy, the production of like or directly competitive articles in the United States, and United States consumers, it finds that such articles should not be excluded from entry." 19 U.S.C. § 1337(d)(l). Similarly, the Commission must consider these public interest factors before issuing a CDO. 19 U.S.C. § 1337(f)(1).

Under appropriate facts and circumstances, the Commission may determine that no remedy should issue because of the adverse impacts on the public interest. *See, e.g.*, *Certain Fluidized Supporting Apparatus & Components Thereof*, Inv. Nos. 337-TA-182/188, USITC Pub. 1667, Comm'n Op. at 1–2, 23–25 (Oct. 1984) (finding that the public interest warranted denying complainant's requested relief). Moreover, when the circumstances of a particular investigation require, the Commission has tailored its relief in light of the statutory public interest factors. For example, the Commission has allowed continued importation for ongoing medical research, exempted service parts, grandfathered certain infringing products, and delayed the imposition of remedies to allow affected third party consumers to transition to non-infringing products. *E.g.*,

Certain Microfluidic Devices, Inv. No. 337-TA-1068 Comm'n Op. at 1, 22–48, 53–54 (analyzing the public interest, discussing applicable precedent, and ultimately issuing a tailored LEO and a tailored CDO); *Certain Road Milling Machines & Components Thereof*, Inv. No. 337-TA-1067, Comm'n Op. at 32–33 (July 18, 2019) (exempting service parts); *Certain Baseband Processor Chips & Chipsets, Transmitter, & Receiver (Radio) Chips, Power Control Chips, & Prods. Containing Same, Including Cellular Tel. Handsets*, 337-TA-543, USITC Pub. No. 4258, Comm'n Op. at 150–51 (Oct. 2011) (grandfathering certain products); *Certain Personal Data & Mobile Comm'n Devices & Related Software*, 337-TA-710, USITC Pub. No. 4331, Comm'n Op., at 72–73, 80–81 (June 2012) (delaying imposition of remedy).

The statute requires the Commission to consider and make findings on the public interest in every case in which a violation is found regardless of the quality or quantity of public interest information supplied by the parties. 19 U.S.C. § 1337(d)(l), (f)(l). Thus, the Commission publishes a notice inviting the parties as well as interested members of the public and interested government agencies to gather and present evidence on the public interest at multiple junctures in the proceeding. 19 U.S.C. § 1337(d)(l) & (f)(l).

On July 25, 2019, the Commission issued a notice soliciting comments on public interest issues raised by the relief recommended in the RD. Notice at 1 (July 25, 2019). No comments from the public were received in response to that notice. On August 26, 2019, pursuant to Commission Rule 210.50(a)(4), 10X and Bio-Rad each submitted briefs addressing the effect the RD's proposed remedies would have on the public interest.[21] The parties also submitted additional public interest arguments with their responses to the Commission's notice of review, and their

[21] Complainant 10X Genomics, Inc.'s Submission on the Public Interest (Aug. 26, 2019) ("10X BPI"); Bio-Rad's Statement on Public Interest (Aug. 26, 2019) ("Bio-Rad BPI").

replies to those responses. The parties' arguments with respect to each of the public interest factors are summarized below.[22]

A. Public Health and Welfare

Concerning the public health and welfare, 10X submitted that "[t]here are no public health, safety, or welfare concerns relating to the requested remedial orders." 10X BPI at 1. 10X also argued that Bio-Rad should not be permitted to argue that remedial orders would adversely affect the public health and welfare in this investigation because it argued that remedial orders in the 1068 investigation would not cause such adverse effects. *See* 10X BPI at 1–2. Further, 10X asserted that [redacted]. *See id.* at 2. 10X substantially reiterated these arguments in its brief responding to the Commission's notice of review. *See* 10X Resp. to Qs. at 60–61.

For its part, Bio-Rad confined itself to arguing that if 10X's public health and welfare arguments in the 1068 investigation justify a modification of the remedy in that investigation then the same arguments should justify a modification in this investigation. *See* Bio-Rad BPI at 3.

On the record of this investigation, the Commission has determined that the public health and welfare will not be adversely affected by issuance of a tailored LEO and a similarly tailored CDO. Of note, the LEO and CDO issued today include exemptions to allow researchers who have been using Bio-Rad's ddSEQ systems in the United States as of the date of the issuance of those orders, and who have provided Bio-Rad a documented need to continue procuring consumables for those systems for an identified current ongoing research project for which that need cannot be

[22] The Commission did not delegate responsibility to the ALJ for taking evidence and making findings concerning the effect of a remedy on the public interest in this investigation.

met by any alternative product, to continue to procure and use such consumables. Bio-Rad's ddSEQ system is used by medical researchers "to study the ways in which individual cells from a tumor differ from each other." Bio-Rad BPI at 1; *see also id.* at 2, n.2 (listing published research that used Bio-Rad's technology). In the 1068 investigation, the Commission considered a large volume of evidence about the adverse effects attendant to disrupting important medical research by forcing researchers to switch instruments mid-study, which Bio-Rad contested. *See* Inv. No. 337-1068, Comm'n Op. at 45–46. On the record of the 1068 investigation, the Commission determined that disruption of such research would adversely affect the public health and welfare to such a degree that the remedial orders in that investigation should include exemptions to allow ongoing research to continue without disruption. *See id.*

The record on the public interest in this investigation is not nearly as robust as the one in the 1068 investigation. As noted, in addressing the public health and welfare, Bio-Rad has merely argued that whatever argument prevails in the 1068 investigation should prevail here as well. *See* Bio-Rad BPI at 3. Bio-Rad's argument suggests that its ddSEQ systems are so comparable to the accused products in the 1068 investigation that any adverse effects attendant to the exclusion of those products must attend the exclusion of its products as well. Bio-Rad has not, however, presented evidence sufficient for the Commission to draw that conclusion, and the Commission does not agree with Bio-Rad's underlying premise that the remedies in the 1068 investigation and this one must be reciprocal because the underlying products have similar uses. Nonetheless, here, unlike Bio-Rad's position in the 1068 investigation, 10X affirmatively proposed an exemption to the remedial orders to allow the use of Bio-Rad's ddSEQ systems in ongoing research to continue. *See* 10X BPI at 1 ("[T]o address any potential public interest concern, 10X does not oppose a limited carveout for sales of consumables imported for sale to customers who have access to

existing instruments in the United States as of the Target Date so that Bio-Rad's current customers with access to existing instruments may continue to perform their research, as well as for warranty support, service, repair, and replacement of existing instruments if such warranty is currently offered and covers such activities.").[23]

Accordingly, as stated above, the Commission has determined to issue an LEO and CDO in this investigation that incorporate 10X's proposed exemptions because the parties have agreed to this remedy.

B. Competitive Conditions in the United States Economy

With respect to competitive conditions, 10X argued that exclusion of Bio-Rad's accused products would have no material impact on competitive conditions in the United States because [redacted], 10X's own products provide similar functionality to Bio-Rad's, and 10X's own products are superior to Bio-Rad's. *See* 10X BPI at 2–3. 10X disputed any suggestion that competitive conditions would be harmed due to the removal of a large supplier from the market because, in 10X's view, [redacted]. *See id.* at 3. 10X further submitted that the introduction of its next generation products will also blunt any detrimental effects to competition that may result from exclusion of its older products in other litigation. *See id.* at 3–4. Finally, 10X asserted that Bio-Rad's assertion in the 1068 investigation that numerous alternatives exist to both 10X and Bio-Rad's products should preclude it from arguing in this investigation no suitable alternatives exist. *See id.* at 4. Here again, 10X substantially reiterated these arguments in its brief responding to the Commission's notice of review. *See* 10X Resp. to Qs. at 61–63, 64–65.

[23] Bio-Rad's arguments regarding availability of 10X's products, and alleged flaws in those products, are addressed below in section XII.C.

Bio-Rad did not specifically identify any adverse effects on competitive conditions in the United States economy that would flow from issuance of remedial orders in this investigation. *See generally* Bio-Rad BPI; Bio-Rad Resp. to Qs. at § XI.C; Bio-Rad Reply at § XI.D.

On the record of this investigation, the Commission has determined that competitive conditions in the United States economy will not be adversely affected by the issuance of the remedial orders in this investigation. Bio-Rad has not rebutted 10X's assertions that [redacted]. Moreover, evidence submitted by 10X shows that Bio-Rad's ddSEQ products appear in only a small number of research publications, which tends to reinforce the conclusion that adoption of Bio-Rad's ddSEQ products has been modest. *See* 10X Resp. to Qs., Ex. I (search results for "ddSEQ" in medical publication database). [redacted], the Commission finds that exclusion of those products will not adversely affect competitive conditions in the United States.

C. Production of Like or Directly Competitive Articles in the United States

10X submitted that "[t]he production of 'like or directly competitive' articles in the United States will not be harmed and may be helped by the recommended orders," because Bio-Rad [redacted] while 10X manufactures consumables and assembles instruments in the United States. 10X BPI at 4. In 10X's view, "[s]ubstituting 10X's products for Bio-Rad's will not harm domestic production and will, if anything, increase it." *Id.*

Bio-Rad disputed 10X's position based on the fact that "10X has been enjoined from selling any of the products it used to establish the domestic industry in this case to new customers." Bio-Rad BPI at 4 (citing *Bio-Rad et al. v. 10X*, No. 1:15-cv-00152-RGA, Dkt. 576 (D. Del. Aug. 12, 2019)). Bio-Rad also pointed to the possibility of an exclusion order in the 1068

investigation.[24] *See id.* Concerning 10X's next generation Next GEM product, Bio-Rad pointed to an SEC filing from 10X calling into question whether the Next GEM chip will be a viable replacement for the GEM chip. *See id.* (citing https://www.sec.gov/Archives/edgar/data/1770787/000119312519224368/d737378ds1.htm at 7). Additionally, Bio-Rad suggested that the Commission should not rely on 10X's own products as possible replacements for Bio-Rad's because 10X's financial stability is uncertain. *See* Bio-Rad BPI at 4–5. Bio-Rad drew support for that suggestion from an SEC filing by 10X discussing the various risks its business currently faces. *See* Bio-Rad BPI at 4–5 (citing https://www.sec.gov/Archives/edgar/data/1770787/000119312519224368/d737378ds1.htm at 15). Finally, Bio-Rad argued that 10X's own arguments in the 1068 investigation regarding the infeasibility of switching its customers to other instruments should apply equally in this investigation to Bio-Rad's customers and instruments. *See id.* at 5.

In response to Bio-Rad's arguments, 10X first argued that neither the district court injunction nor any exclusion order in the 1068 investigation will prevent it from filling the demand created by excluding Bio-Rad's products because 10X's next generation products, which were launched in May 2019, are not subject to either order. *See* 10X Resp. to Qs. at 62. 10X also disputed Bio-Rad's characterization of its next generation products as "unproven." *See id.* at 63. Further, 10X asserted that its transition to its next generation products will not prevent it from being able to meet any demand resulting from exclusion of Bio-Rad's products. *See id.*

Next, 10X disputed Bio-Rad's suggestion that its financial stability would hamper its ability to meet demand for microfluidic systems and components. *See id.* at 64. Particularly, 10X

[24] Since the parties submitted their briefs, an exclusion order and a cease and desist order have issued in connection with the 1068 investigation. *See* 84 Fed. Reg. 70999 (Dec. 26, 2019).

pointed to its initial public offering and its revenue numbers for the first half of 2019 as evidence of its financial stability. *See id.* And finally, cornering the litigation mentioned in its prospectus, 10X acknowledged that there is ongoing litigation related to its next generation products, but submitted that speculation about the outcome of that litigation at some point in the future should not preclude issuance of an exclusion order where a violation has already been proven. *See id.*

In its own response to the Commission's notice of review, Bio-Rad argued that a recently published study demonstrates flaws in 10X's Chromium scATAC-seq assay. *See* Bio-Rad Reply at 58–59. Bio-Rad asserted that the flaws identified in this study are present throughout all of 10X's products, including its next generation product line. *See id.* The thrust of Bio-Rad's point is that 10X's products are not superior to Bio-Rad's, and that the public interest will be harmed if researches are forced to utilize inferior equipment. *See id.* at 59.

The Commission finds Bio-Rad's assertion that 10X will be unable to fill demand created by the exclusion of its ddSEQ products to be speculative. While 10X's domestic industry products may be subject to an exclusion order and an injunction, its next generation products are not. As noted above, an exemption for existing use of ddSEQ products in this investigation, in combination with the similar exemption for 10X's products in the 1068 investigation, will protect the public interest with respect to extant use of those products where switching to a new product would be unworkable. For new uses, the public is free to use 10X's next generation products. Bio-Rad cites no evidence to support its assertions that 10X's next generation products are "unproven" or have "no track record," and therefore the Commission does not credit those assertions. By contrast, 10X produced two white papers supporting its assertion that its next generation products provide comparable performance to its earlier products. *See* 10X Resp. to Qs., Ex. J at 1, 8; Ex. K at 1, 4. While 10X's SEC filings do acknowledge the risks and inherent uncertainty involved in launching

a new product, the statements therein primarily concern 10X's ability to replace its own prior products with its next generation products. *See id.*, Ex. H at 6–7. The filing does not suggest that 10X will be unable to manufacture its next generation products in volumes sufficient to replace [redacted] Bio-Rad's ddSEQ products in use. *See id.*

Bio-Rad points out that 10X's SEC filings acknowledge that one of the risks potential investors should consider is the fact that, as of June 30, 2019, it had accumulated a deficit of $245.6 million. *See* https://www.sec.gov/Archives/edgar/data/1770787/000119312519224368/d737378ds1.htm at 15. However, 10X has since completed its initial public offering with a market capitalization near $5 billion. *See* 10X Resp. to Qs., Ex. L at 1. Thus, while the record evidence indicates that investors in 10X may be subject to some risk based on 10X's revenue and deficits, the Commission finds that it would be speculative at this point to determine that 10X's financial health will hinder it from offering its next generation products to the public. The Commission also finds that the discussion of litigation risk in the SEC filings is similarly speculative. Bio-Rad has identified no litigation currently precluding 10X from offering its next generation products domestically, and the Commission declines to speculate on the outcome of ongoing litigation.

Finally, with respect to Bio-Rad's argument that all of 10X's products are tainted by common flaws, Bio-Rad relied on a publication titled "Inference and effects of barcode multiplets in droplet-based single-cell assays" by Lareau *et al.* and a declaration by Dr. Lior Pachter, a Bio-Rad expert witness from the 1068 investigation. *See* Bio-Rad Reply, Ex. A & Pachter Decl. While the Lareau publication does report flaws associated with 10X's scATAC-seq assay, Bio-Rad Reply, Ex. A at 2, which Dr. Pachter asserts are equally applicable across 10X's entire line of products, *see* Pachter Decl. at ¶ 7, Dr. Pachter also acknowledges in his declaration that 10X is aware of the issue reported in the Lareau publication and that it has published a statement on its

website indicating that the issue in its scATAC product can be corrected with software processing, *see id.* at ¶ 10. Dr. Pachter's declaration reproduces a portion of that statement in which 10X acknowledges the issue identified in the Lareau publication, but omits the portion of the statement in which 10X explains the actions it has or will take to address the issue. *Compare id. with* https://www.10xgenomics.com/blog/letter-from-10x-genomics. Based on the publication and Dr. Pachter's declaration, Bio-Rad concluded that if its products "are excluded and [Bio-Rad's] future potential customers are forced to use 10X systems, their medical research efforts — research which 10X characterizes as very important to public health — will be hampered by 10X's faulty data output." Bio-Rad Reply at 15.

Bio-Rad's conclusion overreaches with respect to what the evidence shows. The underlying publication shows a flaw attendant to 10X's scATAC-seq assay. *See* Bio-Rad Reply, Ex. A at 2. Dr. Pachter's declaration, if accepted as true, supports the conclusion that the underlying flaw is present across all of 10X's single cell product line. *See* Pachter Decl. at ¶¶ 7, 12. However, Dr. Pachter's declaration also supports the conclusion that 10X is aware of the Lareau publication and the issue reported therein, and has devised a method of correcting the issue through computational means. *See id.* at ¶ 10. Though Dr. Pachter stated that "10X Genomics has not released any data or validation demonstrating that their computational solution to eliminating barcode multiplets removes all multiplets, and does not erroneously filter out single barcode cells," *see id.* at 15, that fact is not surprising given the short time between when the publication was published on October 30, 2019, and November 7, 2019, when Dr. Pachter signed his declaration.

The Commission declines to presume that 10X's entire product line is flawed beyond correction based on a publication that does not go so far, and testimony from a declarant who only implies, without support, that the computational correction proposed by 10X will not be effective.

Accordingly, on the record of this investigation, the Commission finds that the issuance of remedial orders in this investigation will not adversely affect the production of like or directly competitive articles in the United States.

D. United States Consumers

10X argued that the proposed remedial orders would have a minimal impact on U.S. consumers due to [redacted] and the fact that, as discussed above, 10X does not oppose exempting existing users of Bio-Rad's ddSEQ instruments from such orders. *See* 10X BPI at 5. As with the other public interest factors, 10X also argued that Bio-Rad's statements in the 1068 investigation to the effect that United States consumers would not be harmed by an exclusion order in that investigation should preclude Bio-Rad from arguing that the proposed remedial orders in this investigation would harm consumers. *See id.* 10X's assertions in its responses to the Commission's notice of review regarding the effect of remedial orders United States consumers are substantially aligned with its arguments in its public interest briefing. *See* 10X Resp. to Qs. at 65–66.

Bio-Rad argued only that "[b]ecause 10X's prior products are subject to an injunction and its new products are unproven, an exclusion order against Bio-Rad's products could force consumers to use noncommercial and unproven technologies to pursue their research objectives." Bio-Rad BPI at 5.

The arguments presented addressing the effect of a remedy on United States consumers are substantially coextensive with the arguments advanced in the context of the other public interest factors. 10X relies on the [redacted] for ddSEQ products to argue that any impact on consumers from their exclusion will be minimal, while Bio-Rad again asserts that 10X's products are already subject to exclusion, or if not are unproven. For reasons similar to those given above, the Commission finds that the evidence in this investigation does not establish that United States

consumers will be harmed by the issuance of a tailored LEO and similarly tailored CDO in this investigation.

E. Commission Determination on Public Interest

Upon consideration of the parties' submissions, and after considering the effect that remedial orders would have on the public interest, the Commission has determined to issue a tailored LEO and a similarly tailored CDO. The exemptions to the LEO and CDO proposed by 10X will allow the work of researchers already using Bio-Rad's products to continue.

XIII. CONCLUSION

For the reasons discussed above, the Commission has determined that Bio-Rad violated Section 337 by importing into the United States, selling for importation, or selling in the United States after importation certain microfluidic systems and components thereof and products containing same by reason of infringement of certain claims of the '024,'468, and '530 patents. The Commission finds no violation with respect to the asserted claims of the '204 patent. The Commission has determined to issue a limited exclusion order and a cease and desist order against Bio-Rad. The Commission finds that the public interest factors do not weigh against issuing these remedial orders. The Commission has further determined that during the Period of Presidential review, a bond in the amount of twenty-five (25) percent of entered value shall be applied to covered Bio-Rad products.

By order of the Commission.

Lisa R. Barton
Secretary to the Commission

Issued: March 24, 2020

CERTAIN MICROFLUIDIC SYSTEMS AND COMPONENTS THEREOF AND PRODUCTS CONTAINING SAME **Inv. No. 337-TA-1100**

PUBLIC CERTIFICATE OF SERVICE

I, Lisa R. Barton, hereby certify that the attached **COMMISSION OPINION** has been served by hand upon the Commission Investigative Attorney, **Monica Bhattacharyya, Esq.,** and the following parties as indicated, on **March 25, 2020.**

Lisa R. Barton, Secretary
U.S. International Trade Commission
500 E Street, SW, Room 112
Washington, DC 20436

On Behalf of Complainants 10X Genomics, Inc.:

Paul T. Ehrlich
TENSEGRITY LAW GROUP LLP
555 Twin Dolphin Dr., Suite 650
Redwood Shores, CA 94061
Email: paul.ehrlich@tensegritylawgroup.com

☐ Via Hand Delivery
☐ Via Express Delivery
☐ Via First Class Mail
☒ Other: Email Notification of Availability for Download

On Behalf of Respondents Bio-Rad Laboratories, Inc.:

S. Alex Lasher
QUINN EMANUEL URQUHART & SULLIVAN, LLP
1300 I Street NW, Suite 900
Washington, DC 20005
Email: alexlasher@quinnemanuel.com

☐ Via Hand Delivery
☐ Via Express Delivery
☐ Via First Class Mail
☒ Other: Email Notification of Availability for Download

PUBLIC VERSION

UNITED STATES INTERNATIONAL TRADE COMMISSION

Washington, D.C.

In the Matter of **CERTAIN MICROFLUIDIC SYSTEMS AND COMPONENTS THEREOF AND PRODUCTS CONTAINING SAME**	**Inv. No. 337-TA-1100**

INITIAL DETERMINATION ON VIOLATION OF SECTION 337

Administrative Law Judge Dee Lord

(July 12, 2019)

Appearances:

For Complainant 10X Genomics, Inc.:

Matthew D. Powers, Esq., Paul T. Ehrlich, Esq., Azra M. Hadzimehmedovic, Esq., Aaron M. Nathan, Esq., and Stefani C. Smith, Esq. of Tensegrity Law Group, LLP of Redwood Shores, CA.

For Respondent Bio-Rad Laboratories, Inc.:

Kevin P.B. Johnson, Esq., Brian Cannon, Esq., and Victoria Maroulis, Esq. of Quinn, Emanuel, Urquhart & Sullivan, LLP of Redwood Shores, CA; S. Alex Lasher, Esq. and Sean Gloth, Esq. of Quinn, Emanuel, Urquhart & Sullivan, LLP of Washington, DC; and David Bilsker, Esq. of Quinn, Emanuel, Urquhart & Sullivan, LLP of San Francisco, CA.

For the Commission Investigative Staff:

Monica Bhattacharyya, Esq. and Anne Goalwin, Esq. of the Office of Unfair Import Investigations.

PUBLIC VERSION

Pursuant to the Notice of Investigation (Dec. 13, 2017) and Commission Rule 210.42, this is the administrative law judge's final initial determination in the matter of *Certain Microfluidic Systems and Components Thereof and Products Containing Same*, Commission Investigation No. 337-TA-1100. 19 C.F.R. § 210.42(a)(1)(i).[1]

For the reasons discussed herein, it is my final initial determination that there is a violation of section 337 of the Tariff Act of 1930, as amended, 19 U.S.C. § 1337, in the importation into the United States, the sale for importation, and/or the sale within the United States after importation of certain microfluidic systems and components thereof and products containing same by reason of infringement of certain claims of U.S. Patent No. 9,689,024 ("the '024 Patent"), U.S. Patent No. 9,695,468 ("the '468 Patent"), and U.S. Patent No. 9,856,530 ("the '530 Patent"). There is no violation with respect to U.S. Patent No. 9,644,204 ("the '204 Patent").

[1] Pursuant to Commission Rule 210.42(a)(1)(ii), a recommended determination on remedy and bonding shall issue within 14 days of this initial determination. 19 C.F.R. § 210.42(a)(1)(ii).

PUBLIC VERSION

TABLE OF CONTENTS

PUBLIC VERSION

The following abbreviations may be used in this Initial Determination:

Tr.	Transcript
WS	Witness Statement
DWS	Direct Witness Statement
RWS	Rebuttal Witness Statement
JX	Joint Exhibit
CX	Complainant's exhibit
CPX	Complainant's physical exhibit
CDX	Complainant's demonstrative exhibit
RX	Respondent's exhibit
RPX	Respondent's physical exhibit
RDX	Respondent's demonstrative exhibit
CPHB	Complainant's pre-hearing brief
CIB	Complainant's initial post-hearing brief
CRB	Complainant's reply post-hearing brief
RPHB	Respondent's pre-hearing brief
RIB	Respondent's initial post-hearing brief
RRB	Respondent's reply post-hearing brief
SPHB	Staff's pre-hearing brief
SIB	Staff's initial post-hearing brief
SRB	Staff's reply post-hearing brief

PUBLIC VERSION

I. BACKGROUND

A. Procedural History

The Commission instituted this investigation in response to a complaint filed by 10X Genomics, Inc. ("10X") alleging violations of section 337 of the Tariff Act of 1930, as amended, by reason of infringement of certain claims of U.S. Patent No. 9,644,204 ("the '204 Patent"), U.S. Patent No. 9,689,024 ("the '024 Patent"), U.S. Patent No. 9,695,468 ("the '468 Patent"), and U.S. Patent No. 9,856,530 ("the '530 Patent") by Respondent Bio-Rad Laboratories, Inc. ("Bio-Rad"). The Commission ordered that an investigation be instituted to determine:

> whether there is a violation of subsection (a)(1)(B) of section 337 in the importation into the United States, the sale for importation, or the sale within the United States after importation of certain microfluidic systems and components thereof and products containing same by reason of infringement of one or more of claims 1-4, 6-9, 17, 20, 21, 23, 25, 27, 29, 31, and 33 of the '204 Patent; claims 1, 2, 5, 8, 10, 11, 13, 15-17, 19, 21, and 22 of the '024 Patent; claims 1-4, 6-9, 11, 12, 21, and 22 of the '468 Patent; and claims 1-6, 8-11, 14-20, and 24-30 of the '530 Patent; and whether an industry in the United States exists as required by subsection (a)(2) of section 337;

Notice of Investigation at 2. The investigation was instituted upon publication of the notice of investigation in the *Federal Register* on Wednesday, February 21, 2018. 83 Fed. Reg. 7491-92 (2018); *see* 19 C.F.R. § 210.10(b). Bio-Rad filed a response to the complaint and notice of investigation on March 6, 2018.

A *Markman* hearing was held in this investigation on July 25, 2018, and a *Markman* order, issued on October 31, 2018. Order No. 22.

On October 5, 2018, 10X's motion for summary determination was granted pursuant to a stipulation between 10X and Bio-Rad that 10X has satisfied the economic prong of the domestic industry requirement. Order No. 19 (Oct. 5, 2018), *not reviewed by* Comm'n Notice (Nov. 6, 2018).

10X withdrew its allegations of infringement with respect to claims 2, 8, 10, 11, 13, 15, 16, and 21 of the '024 patent, claims 1, 2, 3, 4, 6, 7, 8, 9, 17, 20, 21, 23, and 25 of the '204 patent, claims 2, 3, 4, 8, 11, 12, and 22 of the '468 patent, and claims 2, 3, 5, 6, 8, 9, 10, 15, 16, 17, 18, 20, 24, 25, 27, 29, and 30 of the '530 patent. Order No. 26 (Nov. 30, 2018); Order No. 27 (Dec. 10, 2018); Comm'n Notice (Dec. 21, 2018). Part of Bio-Rad's inventorship defense was terminated pursuant to Order No. 34 (Feb. 21, 2019), *not reviewed by* Comm'n Notice (Mar. 13, 2019). The evidentiary hearing proceeded on March 25-29, 2019, and the target date was extended to November 12, 2019, pursuant to Order No. 45 (May 29, 2019), *not reviewed by* Comm'n Notice (Jun. 13, 2019).

B. The Private Parties

1. Complainant

The complainant is 10X Genomics, Inc. ("10X"). Notice of Investigation at 2. 10X was founded in 2012 in Pleasanton, California, where it maintains its headquarters and a manufacturing facility. Complaint ¶ 6 (Jan. 9, 2018); Order No. 19 at 3-4 (Oct. 5, 2018).

2. Respondents

The respondent is Bio-Rad Laboratories, Inc. ("Bio-Rad"). Notice of Investigation at 2. Bio-Rad is a Delaware corporation with its principal place of business in Hercules, California. Response to Complaint ¶ 22 (Mar. 6, 2018).

C. Products at Issue

The products at issue are microfluidic cartridges, droplet generation instruments, and assays used in single-cell sequencing.

1. Domestic Industry

The domestic industry products ("DI products") are 10X's GemCode™ and Chromium™ product lines. Order No. 19 at 3. These products were developed by 10X based on its GEM ("Gel bead in Emulsion") architecture, and the first GemCode™ product was sold in 2015. CX-0003C (Schnall-Levin DWS) at Q/A 47-52. The DI products include both single-cell and linked-read applications, including the Chromium™ Single Cell 3' Solution, Chromium™ Single Cell V(D)J Solution, and GemCode™ Single Cell platform (collectively, "10X's single-cell applications), and the Chromium™ Genome Solution, Chromium™ Exome Solution, Chromium™ *de novo* Assembly Solution, and GemCode™ Long Read platform (collectively, "10X's linked-read applications"). Order No. 19 at 3. Pursuant to Order No. 19, 10X has satisfied the economic prong of the domestic industry requirement with respect to these products. *See* Comm'n Notice (Nov. 6, 2018).

2. Accused Products

The accused products are components and assays of Bio-Rad's ddSEQ system, which includes ddSEQ [redacted]. CIB at 4-5; RIB at 11-12. The ddSEQ v1 products include Bio-Rad's ddSEQ v1 Cartridge, ddSEQ v1 Single-Cell Isolator, ddSEQ Cartridge Holder, and consumables and assays used with and/or as part of Bio-Rad's ddSEQ v1 system including the SureCell WTA 3' v1 assay. *Id.*; CX-0004C (Butte DWS) at Q/A 54; RX-0665C (Metzger RWS) at Q/A 29. The ddSEQ [redacted]. *Id.* Bio-Rad has admitted that each of the ddSEQ v1 instruments and the v1 [redacted]. CX-0041C at Interrogatory Nos. 4 and 5; *see* RPHB at 53.

D. Background of Asserted Patents

1. The '024 and '468 Patents

Through application 13/966,150 ("the '150 application"), which was filed on August 13, 2013, the '468 and '024 patents claim priority to six provisional applications filed between August 14, 2012 and July 10, 2013. '024 patent (JX-0003), cover; '468 patent (JX-0005), cover. The '024 patent was filed as a divisional of the '150 application and the '468 patent was filed as a continuation of the '150 application. '024 patent, cover; '468 patent, cover. Because of their ancestry, the '024 and '468 patents share a common specification. The patents identify Benjamin Hindson, Serge Saxonov, and Michael Schnall-Levin as inventors. '024 patent, cover; '468 patent, cover.

Analysis of biological materials, such as sequencing nucleic acids, requires proper sample preparation. '024 patent, col. 1:28-30. "Sample preparation may . . . involve fragmenting molecules, isolating molecules, and/or attaching unique identifiers to particular fragments of molecules . . . ." *Id.* at col. 1:34-37. A microwell partition capsule array can be used in sample preparation operations. *Id.*, col. 4:28-29. Such a device consists of "an assembly of partitions (*e.g.*, microwells, droplets) that are loaded with microcapsules." *Id.*, col. 4:24-27. The array divides the sample "such that a portion of the sample is present in each partition." *Id.*, col. 4:29-32. Each partition "may include one or more capsules that contain one or more reagents (*e.g.*, enzymes, unique identifiers (*e.g.*, bar codes), antibodies, *etc.*)." *Id.*, col. 4:41-44. A "trigger" can be used to cause the microcapsules to release the reagents into the partitions, so that the reagents come into contact with the subdivided sample. *Id.*, col. 4:44-48.

Microcapsules are used (1) to "provide for the controlled and/or timed release of reagents for sample preparation of an analyte," (2) to control the release and transport of reagents, (3) to

deliver reagents in discrete and definable amounts, (4) to "prevent premature mixing of reagents with the sample," and (5) to ease handling of and limit contact with reagents. *Id.*, col. 6:62-col. 7:13. Microcapsules can be formed using gel beads. *Id.*, col. 9:28-35. Analytes and/or reagents can "be coupled/ immobilized to the interior surface of a gel bead (*e.g.*, the interior accessible via diffusion of an oligonucleotide barcode and/or materials used to generate an oligonucleotide barcode) and/or the outer surface of a gel bead." *Id.*, col. 9:36-42. Release of the analytes or reagents from the microcapsule may be the result of applying a trigger. *Id.*, col. 22:4-6. Various types of stimuli can be used as a trigger, including chemical stimuli, enzymes, light, heat, and magnetic fields. *Id.*, col. 19:43-48, col. 22:4-21.

One sample preparation reagent that can be delivered by a microcapsule is a "molecular barcode." *Id.*, col. 12:9-14. For most applications, such as in the case of the nucleic acid sequencing, analyzing multiple samples simultaneously "substantially decreases the cost of analysis as well as increases through-put of the process." *Id.*, col. 12:33-36. To analyze multiple samples, different samples are pooled together. *Id.*, col. 12:36-39. Before the samples are pooled together, the analytes from each sample are tagged with a unique identifier, known in the art as a "molecular barcode," so that analytes from different samples can be identified and tracked in the pooled sample. *Id.*, col. 12:11-13, col. 12:36-39. Molecular barcodes "may comprise a variety of different forms such as oligonucleotide bar codes, antibodies or antibody fragments, fluorophores, nanoparticles, and other elements or combinations thereof." *Id.*, col. 12:14-17. In nucleic acid sequencing, oligonucleotide barcodes are particularly useful. *Id.*, col. 12:43-44.

2. The '204 Patent

The '204 patent issued on May 9, 2017 from an application filed on February 7, 2014. '204 patent (JX-0001), cover. The '204 patent claims priority to four provisional applications filed between February 8, 2013 and July 10, 2013. The provisional applications to which the '204 patent claims priority are also relied on for priority by the '024 and '468 patents. The patent names Benjamin Hindson, Serge Saxonov, Kevin Ness, Paul Hardenbol, Christopher Hindson, Donald Masquelier, Mirna Jarosz, and Michael Schnall-Levin as inventors. Three of the named inventors—Dr. Hindson, Dr. Saxonov, and Dr. Schnall-Levin—are also the named inventors of the '024 and '468 patents.

The disclosed subject matter of the '204 patent is similar to that of the '024 and '468 patents. As with those patents, the '204 patent is directed to sample preparation methods and discloses "compositions comprising a plurality of capsules, the capsules situated within droplets in an emulsion, wherein the capsules are configured to release their contents into the droplets upon the application of a stimulus." *Id.*, col. 1:42-46. The capsules may contain reagents and/or analytes. *Id.*, col. 1:47-48.

3. The '530 Patent

The '530 patent issued on January 2, 2018 from an application filed on May 5, 2017. '530 patent (JX-0007), cover. Through intervening applications, the '530 patent is a continuation in part of an application filed on February 7, 2014. *Id.* The '530 patent also claims priority to five provisional applications filed between December 14, 2012 and July 10, 2013. *Id.* Four of the provisional applications to which the '204 patent claims priority are also relied on for priority by the '024, '468, and '204 patents. The patent names Benjamin Hindson, Serge Saxonov, Kevin Ness, Paul Hardenbol, Mirna Jarosz, and Michael Schnall-Levin as inventors.

These same individuals are named inventors of the '204 patent and three of them—Dr. Hindson, Dr. Saxonov, and Dr. Schnall-Levin—are also named inventors of the '024 and '468 patents.

The claimed subject matter of the '530 patent is similar to the subject matter disclosed in the '024, '468, and '204 patents. As with those patents, the '530 patent discloses sample preparation methods that use microcapsules and beads to provide reagents and analytes in response to stimuli. '530 patent, col. 23:60-col. 24:13.

E. Level of Ordinary Skill in the Art

In the *Markman* order, I adopted Bio-Rad's proposed definition for the level of ordinary skill in the art: either a Ph.D. in molecular biology, molecular genetics, chemistry, engineering, or equivalent disciplines with two years of experience or [B.S.] in such fields with five years of experience, with such experience including library preparation methods, microfluidic technology, and/or bead attachment chemistries. Order No. 22 at 2-3.

F. Witness Testimony

I received testimonial evidence in this investigation in the form of witness statements, live testimony, and deposition designations.

1. Fact Witnesses

10X began the hearing with the testimony of Benjamin Hindson, co-founder of 10X and co-inventor of the asserted patents. CX-0001C; CX-1828C; Tr. 132-187. The next witness was Michael Schnall-Levin, a vice president at 10X and another co-inventor of the asserted patents. CX-0003C; CX-1830C; Tr. 189-231. 10X also called Serge Saxonov, its CEO and also a co-founder of 10X and co-inventor of the asserted patents. CX-1829C; Tr. 768-820.

Bio-Rad presented the testimony of Annette Tumolo, the president of its Life Sciences Group. RX-0502C; Tr. 509-511. Bio-Rad also presented the testimony of Douglas Greiner, a

senior manager in its product development group. RX-0507C; RX-0727C; Tr. 511-565. Bio-Rad also called another of its employees, Nicholas Heredia, who is an alleged co-inventor of the asserted patents. RX-0504C; Tr. 565-604. In addition, Bio-Rad presented the testimony of one of its former employees, Kelly Kaihara (RX-0506C), and she was examined as an adverse witness by 10X. Tr. 234-282. Bio-Rad also presented the testimony of its employee Jeremy Agresti (RX-0503C), who was further examined as an adverse witness by 10X. Tr. 283-348.

2. Expert Witnesses

10X's expert on infringement is Atul Butte, whose testimony was qualified as that of an expert in the field of genomic sequencing solutions. CX-0004C; Tr. 351-474 (expert qualification at 364:9-17). 10X's expert on invalidity is Paul Dear, whose testimony was qualified as that of an expert in the field of genomic sequencing solutions. CX-1827C; Tr. 822-934 (expert qualification at 828:20-829:1).

Bio-Rad's technical expert is Michael Metzker, whose testimony was qualified as that of an expert in next generation sequencing, including sample preparation technologies, microfluidics, enzyme chemistry, high throughput assays, bead properties and attachment chemistries. RX-0664C; RX-0665C; Tr. 608-767 (expert qualification at 613:22-614:14), 935-961.

The parties also stipulated to the admission of witness statements from Thomas Vander Veen (CX-0005C) and Ryan Herrington (RX-0666C), and their designated deposition transcripts (JX-0162C and JX-0170C), discussing the issues of remedy and bond. Tr. 24-25.

3. Deposition Designations

10X submitted designated deposition transcripts for several witnesses: Jeremy Agresti (CX-0009C), Mark DiPanfilo (CX-0010C), Lucas Frenz (CX-0011C), Jodi Goodrich (CX-

0012C), Nicholas Heredia (CX-0014C and CX-0015C), Kelly Kaihara (CX-0016C), Ronald Lebofsky (CX-0018C), Dan Norton (CX-0019C), Carolyn Reifsnyder (CX-0020C), Annette Tumolo (CX-0022C), and Svilen Tzonev (CX-0023C). Tr. 23.

Bio-Rad also submitted designated deposition transcripts for several witnesses: Paul Hardenbol (RX-0396C), Benjamin Hindson (RX-0399C), Christopher Hindson (RX-0400C), Mirna Jarosz (RX-0401C), Donald Masquelier (RX-0405C), Kevin Ness (RX-0408C), Serge Saxonov (RX-0412C), and Michael Schnall-Levin (RX-0413C). Tr. 23-24.

II. JURISDICTION

In order to have the power to decide a case, a court or agency must have both subject matter jurisdiction and jurisdiction over either the parties or the property involved. 19 U.S.C. § 1337; *Certain Steel Rod Treating Apparatus and Components Thereof*, Inv. No. 337-TA-97, Commission Memorandum Opinion, 215 U.S.P.Q. 229, 231 (1981).

A. Subject Matter Jurisdiction

Section 337 confers subject matter jurisdiction on the Commission to investigate, and if appropriate, to provide a remedy for, unfair acts and unfair methods of competition in the importation, the sale for importation, or the sale after importation of articles into the United States. *See* 19 U.S.C. §§ 1337(a)(1)(B) and (a)(2). The Commission has subject matter jurisdiction over this investigation based on 10X's allegation that Bio-Rad has imported the accused products. *Amgen Inc. v. Int'l Trade Comm'n*, 902 F.2d 1532, 1536 (Fed. Cir. 1990). Bio-Rad does not contest the Commission's subject matter jurisdiction in this investigation. RPHB at 53.

B. Personal Jurisdiction

Bio-Rad does not contest the Commission's *in personam* jurisdiction in this investigation. RPHB at 49. Bio-Rad has submitted to the personal jurisdiction of the Commission by answering the Complaint and Notice of Investigation, participating in discovery, appearing at hearings, and filing motions and briefs. *See Certain Miniature Hacksaws*, Inv. No. 337-TA-237, USITC Pub. No. 1948, Initial Determination at 4, 1986 WL 379287, *1 (Oct. 15, 1986), *not reviewed in relevant part by* Comm'n Action and Order, 1987 WL 450871 (Jan. 15, 1987).

C. *In Rem* Jurisdiction

The Commission has *in rem* jurisdiction over the accused products by virtue of their importation into the United States. *See Sealed Air Corp. v. U.S. Int'l Trade Comm'n*, 645 F.2d 976, 985-86 (C.C.P.A. 1981) (holding that the ITC's jurisdiction over imported articles is sufficient to exclude such articles). Bio-Rad does not contest that it has imported, sold for importation, and/or sold after importation certain ddSEQ products. RPHB at 53.

III. LEGAL STANDARDS

A. Infringement

Section 337(a)(1)(B)(i) prohibits "the importation into the United States, the sale for importation, or the sale within the United States after importation by the owner, importer, or consignee, of articles that – (i) infringe a valid and enforceable United States patent or a valid and enforceable United States copyright registered under title 17." 19 U.S.C. §1337(a)(1)(B)(i). The Commission has held that the word "infringe" in Section 337(a)(1)(B)(i) "derives its legal meaning from 35 U.S.C. § 271, the section of the Patent Act that defines patent infringement." *Certain Electronic Devices with Image Processing Systems, Components Thereof, and Associated Software*, Inv. No. 337-TA-724, Comm'n Op. at 13-14 (December 21, 2011). Under 35 U.S.C. § 271(a), direct infringement of a patent consists of making, using, offering to sell, or

selling the patented invention without consent of the patent owner.

In addition to direct infringement, a respondent may be liable for indirect infringement, including induced infringement, which is defined in section 271(b) of the Patent Act: "Whoever actively induces infringement of a patent shall be liable as an infringer." 35 U.S.C. § 271(b). *See DSU Med. Corp. v. JMS Co., Ltd.*, 471 F.3d 1293, 1305 (Fed. Cir. 2006) (*en banc*) ("To establish liability under section 271(b), a patent holder must prove that once the defendants knew of the patent, they actively and knowingly aided and abetted another's direct infringement.") (citations omitted). "The mere knowledge of possible infringement by others does not amount to inducement; specific intent and action to induce infringement must be proven." *Id.* (citations omitted). The Federal Circuit has held that induced infringement "requires knowledge that the induced acts constitute . . . infringement." *Global-Tech Appliances, Inc. v. SEB S.A.*, 563 U.S. 754, 766 (2011). In *Suprema, Inc. v. Int'l Trade Comm'n*, the Federal Circuit upheld the Commission's interpretation of the section 337 language "articles that infringe" in the context of induced infringement, holding that the statute "covers goods that were used by an importer to directly infringe post-importation as a result of the seller's inducement." 796 F.3d 1338, 1352-53 (Fed. Cir. 2015).

Another form of indirect infringement is contributory infringement, defined in section 271(c) of the Patent Act: "Whoever offers to sell . . . or imports into the United States a component of a patented machine, . . . or a material or apparatus for use in practicing a patented process, constituting a material part of the invention, knowing the same to be especially made or especially adapted for use in an infringement of such patent, and not a staple article or commodity of commerce suitable for substantial noninfringing use, shall be liable as a contributory infringer." 35 U.S.C. § 271(c). The intent requirement for contributory

infringement requires that respondent knows "that the combination for which [the] component was especially designed was both patented and infringing." *Global-Tech Appliances, Inc. v. SEB S.A.*, 563 U.S. at 763. A violation of section 337 based on contributory infringement requires that "the accused infringer imported, sold for importation, or sold after importation within the United States, the accused components that contributed to another's direct infringement." *Spansion, Inc. v. Int'l Trade Comm'n*, 629 F.3d 1331, 1353 (Fed. Cir. 2010).

"An infringement analysis entails two steps. The first step is determining the meaning and scope of the patent claims asserted to be infringed. The second step is comparing the properly construed claims to the device accused of infringing." *Markman v. Westview Instruments, Inc.*, 52 F.3d 967, 976 (Fed. Cir. 1995) (*en banc*), *aff'd*, 517 U.S. 370 (1996) (citation omitted). Infringement must be proven by a preponderance of the evidence. *SmithKline Diagnostics, Inc. v. Helena Labs. Corp.*, 859 F.2d 878, 889 (Fed. Cir. 1988). A preponderance of the evidence standard "requires proving that infringement was more likely than not to have occurred." *Warner-Lambert Co. v. Teva Pharm. USA, Inc.*, 418 F.3d 1326, 1341 n.15 (Fed. Cir. 2005).

A complainant must prove either literal infringement or infringement under the doctrine of equivalents. Literal infringement requires the patentee to prove that the accused device contains each and every limitation of the asserted claim(s). *Frank's Casing Crew & Rental Tools, Inc. v. Weatherford Int'l, Inc.*, 389 F.3d 1370, 1378 (Fed. Cir. 2004). "If even one limitation is missing or not met as claimed, there is no literal infringement." *Elkay Mfg. Co. v. EBCO Mfg. Co.*, 192 F.3d 973, 980 (Fed. Cir. 1999). Literal infringement is a question of fact. *Finisar Corp. v. DirecTV Grp., Inc.*, 523 F.3d 1323, 1332 (Fed. Cir. 2008).

B. Invalidity

It is the respondents' burden to prove invalidity, and the burden of proof never shifts to the patentee to prove validity. *Scanner Techs. Corp. v. ICOS Vision Sys. Corp. N.V.*, 528 F.3d 1365, 1380 (Fed. Cir. 2008). "Under the patent statutes, a patent enjoys a presumption of validity, *see* 35 U.S.C. § 282, which can be overcome only through facts supported by clear and convincing evidence . . . ." *SRAM Corp. v. AD-II Eng'g, Inc.*, 465 F.3d 1351, 1357 (Fed. Cir. 2006); *see also Microsoft Corp. v. i4i Ltd. P'ship*, 564 U.S. 91, 100-114 (2011) (upholding the "clear and convincing" standard for invalidity).

The clear and convincing evidence standard placed on the party asserting an invalidity defense requires a level of proof beyond the preponderance of the evidence. Although not susceptible to precise definition, "clear and convincing" evidence has been described as evidence that produces in the mind of the trier of fact "an abiding conviction that the truth of a factual contention is 'highly probable.'" *Price v. Symsek*, 988 F.2d 1187, 1191 (Fed. Cir. 1993) (quoting *Buildex, Inc. v. Kason Indus., Inc.*, 849 F.2d 1461, 1463 (Fed. Cir. 1988)).

1. Anticipation

Pursuant to 35 U.S.C. § 102, a patent claim is invalid as anticipated if:

> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant;
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of the application for patent in the United States;
>
> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent;
>
> (g)(2) before such person's invention thereof, the invention was made in this country by another inventor who had not abandoned, suppressed, or concealed it.

35 U.S.C. § 102 (2000).[2] "A patent is invalid for anticipation if a single prior art reference discloses each and every limitation of the claimed invention. Moreover, a prior art reference may anticipate without disclosing a feature of the claimed invention if that missing characteristic is necessarily present, or inherent, in the single anticipating reference." *Schering Corp. v. Geneva Pharm., Inc.*, 339 F.3d 1373, 1377 (Fed. Cir. 2003) (citations omitted).

2. Obviousness

Section 103 of the Patent Act states:

> A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

35 U.S.C. § 103(a) (2000).[3]

"Obviousness is a question of law based on underlying questions of fact." *Scanner Techs.*, 528 F.3d at 1379. The underlying factual determinations include: "(1) the scope and content of the prior art, (2) the level of ordinary skill in the art, (3) the differences between the claimed invention and the prior art, and (4) objective indicia of non-obviousness." *Id.* (citing *Graham v. John Deere Co.*, 383 U.S. 1, 17-18 (1966)). These factual determinations are often referred to as the "*Graham* factors."

The critical inquiry in determining the differences between the claimed invention and the prior art is whether there is a reason to combine the prior art references. *KSR Int'l Co. v. Teleflex*

---

[2] As explained in the revision notes and legislative reports in 35 U.S.C.A. § 100 (May 13, 2015), the language of 35 U.S.C. § 102 that was effective prior to the America Invents Act controls in this investigation.

[3] *See supra*, n.2.

Inc., 550 U.S. 398, 418-21 (2007). In *KSR*, the Supreme Court rejected the Federal Circuit's rigid application of the teaching-suggestion-motivation test. While the Court stated that "it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does," it described a more flexible analysis:

> Often, it will be necessary for a court to look to interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art, all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed by the patent at issue . . . . As our precedents make clear, however, the analysis need not seek out precise teachings directed to the specific subject matter of the challenged claim, for a court can take account of the inferences and creative steps that a person of ordinary skill in the art would employ.

Id. at 418. Applying *KSR*, the Federal Circuit has held that, where a patent challenger contends that a patent is invalid for obviousness based on a combination of prior art references, "the burden falls on the patent challenger to show by clear and convincing evidence that a person of ordinary skill in the art would have had reason to attempt to make the composition or device . . . and would have had a reasonable expectation of success in doing so." *PharmaStem Therapeutics, Inc. v. ViaCell, Inc.*, 491 F.3d 1342, 1360 (Fed. Cir. 2007).

In addition to demonstrating that a reason exists to combine prior art references, the challenger must demonstrate that the combination of prior art references discloses all of the limitations of the claims. *Hearing Components, Inc. v. Shure Inc.*, 600 F.3d 1357, 1373-1374 (Fed. Cir. 2010), *abrogated on other grounds by Nautilus, Inc. v. Biosig Instruments, Inc.*, 572 U.S. 898 (2014) (upholding finding of non-obviousness based on substantial evidence that the asserted combination of references failed to disclose a claim limitation); *Velander v. Garner*, 348

F.3d 1359, 1363 (Fed. Cir. 2003) (explaining that a requirement for a finding of obviousness is that "all the elements of an invention are found in a combination of prior art references").

C. Domestic Industry

In patent-based proceedings under section 337, a complainant must establish that an industry "relating to the articles protected by the patent . . . exists or is in the process of being established" in the United States. 19 U.S.C. § 1337(a)(2). Under Commission precedent, the domestic industry requirement of section 337 consists of an "economic prong" and a "technical prong." *See, e.g., Alloc, Inc. v. Intl Trade Comm'n,* 342 F.3d 1361, 1375 (Fed. Cir. 2003). To meet the technical prong, the complainant must establish that it practices at least one claim of the asserted patent. *Certain Point of Sale Terminals and Components Thereof*, Inv. No. 337-TA-524, Order No. 40 at 17-18 (Apr. 11, 2005). "The test for satisfying the 'technical prong' of the industry requirement is essentially [the] same as that for infringement, *i.e.*, a comparison of domestic products to the asserted claims." *Alloc*, 342 F.3d at 1375.

With respect to the "economic prong," subsection (3) of Section 337(a) provides:

> For purposes of paragraph (2), an industry in the United States shall be considered to exist if there is in the United States, with respect to the articles protected by the patent, copyright, trademark, mask work, or design concerned –
>
> (A) significant investment in plant and equipment;
>
> (B) significant employment of labor or capital; or
>
> (C) substantial investment in its exploitation, including engineering, research and development, or licensing.

19 U.S.C. § 1337(a)(3).

IV. THE '024 PATENT

A. Asserted Claims

PUBLIC VERSION

10X is asserting claims 1, 5, 17, 19, and 22 of the '024 patent. Claim 1 is independent and the remaining claims depend directly or indirectly from claim 1. Claim 1 recites:

> A method for sample preparation, comprising:
>
> a) providing a droplet comprising a porous gel bead and a target nucleic acid analyte, wherein said porous gel bead comprises at least 1,000,000 oligonucleotide molecules comprising barcode sequences, wherein said oligonucleotide molecules are releasably attached to said porous gel bead, wherein said barcode sequences are the same sequence for said oligonucleotide molecules;
>
> b) applying a stimulus to said porous gel bead to release said oligonucleotide molecules from said porous gel bead into said droplet, wherein upon release from said porous gel bead, a given oligonucleotide molecule from said oligonucleotide molecules attaches to said target nucleic acid analyte; and
>
> c) subjecting said given oligonucleotide molecule attached to said target nucleic acid analyte to nucleic acid amplification to yield a barcoded target nucleic acid analyte.

'024 patent (JX-0003), col. 33:56-col. 34:7.

Claims 5 and 19 depend directly from claim 1. Claim 5 requires that the stimulus applied to the gel bead be "selected from the group consisting of a biological stimulus, a chemical stimulus, a thermal stimulus, an electrical stimulus, a magnetic stimulus, and a photo stimulus." *Id.*, col. 34:15-19. Claim 19 requires that the oligonucleotide molecules attach to the target nucleic acid analytes by hybridization. *Id.*, col. 34:.65-67. Claim 17 depends on claim 16, which requires that the droplet "comprise[] a plurality of target nucleic acid analytes, which plurality of target nucleic acid analytes comprises said target nucleic acid analyte." *Id.*, col. 34:54-56. Claim 17 requires that each of the plurality of target nucleic acid analytes attach to one of the oligonucleotide molecules. *Id.*, col. 34:58-61. Claim 22 depends on claim 21, which requires that the gel bead be formed from polymer gel. *Id.*, col. 35:4-5. Claim 22 requires that the polymer gel be a polyacrylamide. *Id.*, col. 35:6-7.

B. Claim Construction

The parties agreed to construe "barcode" to mean a "label that may be attached to an analyte to convey identifying information about the analyte." Order No. 22 at 2. They agreed to construe "applying a stimulus to said porous gel bead to release said oligonucleotide molecules from said porous gel bead into said droplet" to have its plain and ordinary meaning. *Id.* In the *Markman* order, "1,000,000 oligonucleotides comprising barcode sequences" was construed to mean "1,000,000 oligonucleotide molecules that include, but are not necessarily limited to, barcode sequences." *Id.* at 17-22. The term "releasably attached" was construed to mean "attached in a manner that allows the attached object to be released." *Id.* at 22-30. The terms "amplify" and "amplification" were construed to mean "increasing the number of copies of the target sequence to be detected," including by reverse transcription and without requiring amplification to be performed in a droplet. *Id.* at 31-45.

C. Infringement

10X accuses Bio-Rad's ddSEQ system (v1 [redacted]) of infringing claims 1, 5, 17, 19, and 22 of the '024 patent.

1. Claim 1

There is no dispute that the ddSEQ system includes a method of sample preparation, as recited in the preamble of claim 1, and 10X relies on Dr. Butte's testimony to identify steps corresponding to each limitation. CX-0004C at Q/A 109-226.

a. "providing a droplet . . ."

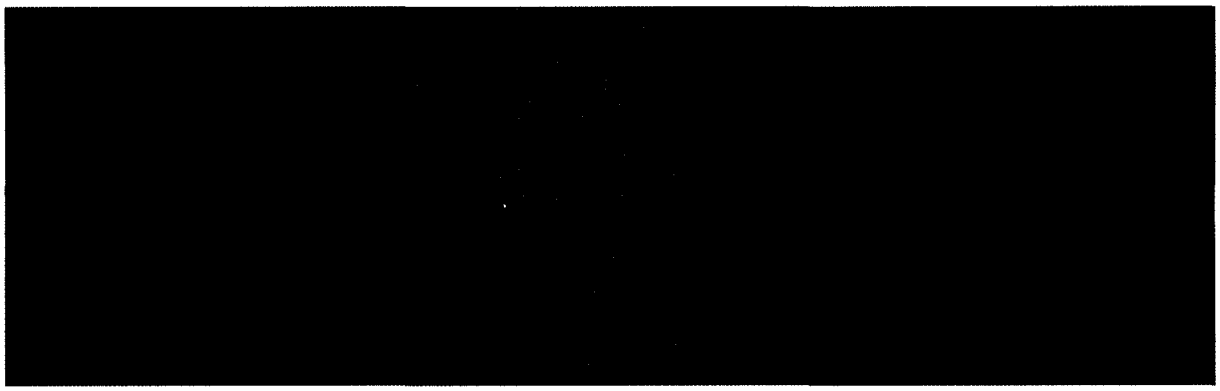
There is no dispute with respect to a majority of the elements in the first limitation of claim 1, which requires "providing a droplet comprising a porous gel bead and a target nucleic acid analyte," wherein the porous gel bead has certain characteristics. CIB at 8-19; SIB 24-29.

Dr. Butte identifies [redacted] gel beads used by the ddSEQ system, which are "porous because each bead has a three-dimensional network of pores [redacted] CX-0004C at Q/A 116. He identifies the steps of making droplets in the ddSEQ v1 workflow, *Id.* at Q/A 61-66, and the [redacted]. *Id.* at Q/A 73-80. He further identifies a targeted nucleic acid analyte: mRNA from a cell or a genomic DNA fragment. *Id.* at Q/A 116-17, 120-23. Bio-Rad does not dispute 10X's allegations with respect to the "porous gel bead" or "nucleic acid analyte."

The claim limitation further requires that the porous gel bed "comprises at least 1,000,000 oligonucleotide molecules comprising barcode sequences," and Dr. Butte cites Bio-Rad documents for the ddSEQ v1 process describing a concentration of oligonucleotides in a droplet with a volume consistent with [redacted]. CX-0004C at Q/A 128 (citing JX-0050C). This is consistent with Dr. Agresti's deposition testimony, where he was asked, "How many oligo molecules are attached to each gel bead in ddSEQ?" CX-0009C at 434. He answered: [redacted] *Id.* [redacted] CX-0004C at Q/A 133 (citing JX-0090C; CX-1529C). Dr. Agresti confirmed that the number of oligonucleotides in the scATAC-seq is [redacted] CX-0009C at 436-37. Bio-Rad disputed this limitation in its pre-hearing brief, RPHB at 57, but does not raise this argument it its post-hearing briefs. *See* RIB at 50-68; RRB at 5-14.

The next element of this limitation requires that "said oligonucleotide molecules are releasably attached to said porous gel bed." As discussed above, "releasably attached" was construed to mean "attached in a manner that allows the attached object to be released." *Id.* at

22-30. There is no dispute that the oligonucleotide molecules in Bio-Rad's ddSEQ system are attached to the gel bead. *See* RRB at 5-6. 10X relies on Dr. Butte's opinion that the oligonucleotide molecules are "releasably attached" to the gel bead through a "linker" that is [redacted] CX-0004C at Q/A 143-45 (citing JX-0050C.00026). Bio-Rad's expert, Dr. Metzker, explains that the [redacted] in the oligonucleotides of the accused products, [redacted]:



RX-0665C at Q/A 78-79 (citing JX-0087.00005).

10X contends that [redacted] in the accused ddSEQ system shows that the oligonucleotide molecules are "releasably attached." CIB at 12-15. There is no dispute that an oligonucleotide molecule containing barcode sequences is released after [redacted] Staff agrees that this process shows that the accused products meet the "releasably attached" limitation. SIB at 26-29.

Bio-Rad argues that its products do not meet the "releasably attached" limitation because [redacted] are part of a long oligonucleotide molecule that contains the barcode sequences and is attached to the gel bead. RIB at 54-59. When [redacted] are removed by [redacted] part of the long oligonucleotide molecule is thus destroyed, and Bio-Rad argues that destroying part of an oligonucleotide molecule is inconsistent with the claim language requiring

that the "oligonucleotide molecules are releasably attached." RIB at 54-59.

In reply, 10X explains that the accused "oligonucleotide molecule" is the molecule that is released [redacted], and no portion of this molecule is destroyed when it is released. CRB at 2. Relying on the opinions of Dr. Butte, 10X divides the Bio-Rad's long oligonucleotide into an accused "oligonucleotide molecule" and a separate "linker."

[redacted]

CRB at 2; CX-0004C (Butte DWS) at Q/A 143-45. Bio-Rad argues that there is no basis for dividing the oligonucleotide in this way, RRB at 5-8, but there are examples of linkers described in the specification. '024 patent, col. 9:57-58 (identifying "chemical linkers"); *see* SIB at 28. Moreover, there is nothing that precludes the claimed "oligonucleotide molecule" from being part of a larger oligonucleotide molecule—as recognized in the *Markman* order, the claim's "recital of 'oligonucleotide molecules' without a qualifier encompasses both larger and smaller oligonucleotide molecules." Order No. 22 at 19. Bio-Rad fails to identify any intrinsic or extrinsic evidence that precludes 10X from identifying the accused portion of Bio-Rad's oligonucleotide as the claimed "oligonucleotide molecule." The claim only requires that the accused "oligonucleotide molecule" include the claimed barcode sequences and that it be released—the molecule identified by 10X meets these limitations.

Bio-Rad further argues that the construction of "releasably attached" requires a reversible process, citing a discussion of the prosecution history in the *Markman* order. RIB at 54-57; RRB at 8. The portion of the *Markman* order cited by Bio-Rad does not support the

imposing a "reversible" limitation on the claims, however. At the *Markman* hearing, Bio-Rad had proposed a construction for "releasably attached" that required the gel bead to be configured or designed to release the attached molecules. Order No. 22 at 22-23. The *Markman* order rejected Bio-Rad's proposed construction for several reasons, including a discussion of the prosecution history where the applicants identified "reversible immobilization" as an example of releasable attachment. *Id.* at 26-27, 29. The discussion of reversible immobilization was cited an example to show that the claims "encompass[] situations wherein a barcode molecule is released from a bead by severing a portion of the barcode molecule," *Id.* at 29, and nothing in the *Markman* order requires that every releasable attachment be reversible. Moreover, limiting "releasably attached" to reversible immobilization would be inconsistent with the claims of the '024 patent, because dependent claim 15 adds "reversibly immobilized" as a limitation. *See Phillips*, 415 F.3d at 1315 ("[T]he presence of a dependent claim that adds a particular limitation gives rise to a presumption that the limitation in question is not present in the independent claim."). Bio-Rad's proposed "reversible" limitation is not consistent with the claims, specification, or prosecution history of the '024 patent.

There is no dispute with respect to the final element of the gel bead limitation, requiring that "said barcode sequences are the same sequence for said oligonucleotide molecules." Dr. Butte identifies documentation for ddSEQ v1 describing the same barcodes for each oligonucleotide in a bead. CX-0004C at Q/A 177-79. In particular, [redacted] CX-0149C.00019. Dr. Butte explains that for Bio-Rad's products to perform their intended purpose, "the barcode sequence should be the same for all the oligonucleotide molecules on a gel bead." CX-0004C at Q/A 180. [redacted]

[redacted]

JX-0091C.0009, .00011. In addition, Bio-Rad employee Dr. Lebofsky confirmed at his deposition that in the scATAC-seq assay, a single bead will have the same barcode on all of the single-stranded DNA fragments on it. CX-0018C at 208:11-15.

Accordingly, the accused ddSEQ v1 [redacted] processes infringe the "providing a droplet . . ." limitation of claim 1 of the '024 patent.

b. "applying a stimulus . . ."

The second limitation of claim 1 of the '024 patent requires "applying a stimulus to said porous gel bead to release said oligonucleotide molecules from said porous gel bead into said droplet." Dr. Butte identifies the [redacted] as the claimed stimulus, which causes the release of the oligonucleotide molecules from the porous gel bead. CX-0004C at Q/A 181-83.

Bio-Rad argues that the accused products do not infringe this limitation because the [redacted] acts on the oligonucleotides, not the gel bead. RIB at 59-62. There is no dispute that the [redacted] acts on the oligonucleotide, but 10X and Staff argue that this is a distinction without a difference, because the oligonucleotide is part of the gel bead. CIB at 19-22; SIB at 29-31. For the reasons discussed below, I agree with 10X and Staff that a preponderance of the evidence shows that the [redacted] is applied to the gel bead in the accused products, and a stimulus that acts on the oligonucleotides attached to the gel bead is consistent with infringement of the "applying a stimulus" limitation.

Dr. Butte explains that the [redacted] is part of an aqueous solution that is applied to the gel bead in the accused products. CX-0004C at Q/A 184. One of Bio-Rad's witness, Dr. Agresti, admitted at his deposition that "in ddSEQ, [] the [redacted] enters the entire volume of the bead." CX-0009C at 343:12-13. As discussed above, the [redacted]

[redacted], releasing the oligonucleotide molecule. *Id.* There is no dispute that the [redacted] thus acts as the stimulus that releases the oligonucleotide molecule from the gel bead into the droplet.

Bio-Rad argues that the [redacted] is applied to the oligonucleotide, not the gel bead; but in the accused products, the oligonucleotides are part of the gel bead. Any stimulus applied to the oligonucleotide is therefore also applied to the gel bead. As Dr. Agresti admitted at the hearing, the oligonucleotides are "[i]nside the volume" of the beads. Tr. 289. *See also* CX-00011C (Frenz Dep. Tr.) at 59-60 (describing oligos "in the volume of the . . . bead."). Bio-Rad cites the language of the '024 patent to argue that the oligonucleotide molecules and gel beads are separately claimed structures, but the claim language explicitly describes the oligonucleotide molecules as a part of the gel beads: "said porous gel bead comprises at least about 1,000,000 oligonucleotide molecules." *See* SIB at 30; SRB at 8. As stated in the *Markman* Order, "[t]he plain and ordinary meaning of 'comprise' is 'to include esp. with a particular scope,' 'to be made up of,' 'compose,' or 'constitute.'" Order No. 22 at 17-18. Recognizing that the oligonucleotides are part of the gel beads is consistent with structure of the accused products and with the language of the asserted claims.

There is no dispute with respect to the remaining elements of this limitation. Dr. Butte identifies a target nucleic acid analyte in the ddSEQ v1 [redacted] "wherein upon release from said porous gel bead, a given oligonucleotide molecule from said oligonucleotide molecules attaches to said target nucleic acid analyte." CX-0004C at Q/A 198 (ddSEQ v1: describing hybridization between the poly-T sequence of the oligonucleotide molecule and the

poly-A tail of the mRNA), Q/A 199 ([redacted]), Q/A 200 (scATAC-seq and [redacted]: Nextera adaptor binding sequence).

c. ". . . nucleic acid amplification"

The third and final limitation of claim 1 of the '024 patent requires "subjecting said given oligonucleotide molecule attached to said target nucleic acid analyte to nucleic acid amplification to yield a barcoded target nucleic acid analyte." As discussed above, the term "amplification" was construed to mean "increasing the number of copies of the target sequence to be detected," including by reverse transcription. Order No. 22 at 31-45.

10X relies on Dr. Butte's analysis of reverse transcription in the accused products to show infringement of this limitation. CIB at 24-28. In the ddSEQ v1 products, Dr. Butte explains that an oligonucleotide molecule attached to mRNA is "subjected to reverse transcription, second strand synthesis, and further PCR, to yield a barcoded cDNA strand." CX-0004C at Q/A 203. He further explains that barcoded cDNA strands are generated from the oligonucleotide molecules through several different processes, which 10X identifies in its brief as four types of amplification. CIB at 24-26 (citing CX-0004C at Q/A 205). In "Type A" the oligonucleotide-mRNA hybrids are subjected to reverse transcription to generate barcoded first cDNA strands. *Id.* In "Type B" the hybrids are subjected to second strand synthesis and further PCR to generate additional barcoded cDNA strands outside the droplet. *Id.* In "Type C" the oligonucleotide molecule attaches to the mRNA through reverse transcription to form the first barcoded cDNA strand, and this cDNA strand is subjected to second strand synthesis outside the droplet to create a second strand of cDNA. *Id.* In "Type D" the first cDNA strand is subjected

to PCR to generate additional double-stranded cDNA outside the droplet. *Id.* In Dr. Butte's opinion, any of these processes would meet the "amplification" limitation of the '024 patent.

Bio-Rad argues that the oligonucleotide molecule only acts as a primer during the reverse transcription reaction, and that this limitation is not infringed because the oligonucleotide molecule itself is not subjected to amplification. RIB at 62-63 (citing RX-0665C (Metzker RWS) at Q/A 87). This interpretation of the claim language was rejected in the *Markman* order, however, which recognized that persons of ordinary skill in the art would understand "amplification" to include reverse transcription. Order No. 22 at 32-41. Notably, the construction of "amplification" does not require exact copies of the oligonucleotide barcodes—the product of amplification can be complementary copies, which are the result of reverse transcription. *Id.* at 35-41. Moreover, dependent claims of the '024 patent explicitly discuss the usage of the oligonucleotide molecule as a primer during amplification. See '024 patent, claim 8 ("The method of claim 1, wherein said given oligonucleotide molecule of said oligonucleotide molecules comprises a region which functions as a primer during said nucleic acid amplification in c)."), claim 10 ("The method of claim 8, wherein said primer is configured to amplify said target nucleic acid analyte."). Bio-Rad's non-infringement argument is not consistent with the claim language of the '024 patent, as construed in this investigation.

Accordingly, the accused ddSEQ v1 [redacted] systems infringe all of the limitations of claim 1 of the '024 patent.

2. Dependent Claims

There is no dispute with respect to the infringement of any the limitations added by dependent claims 5, 17, 19, and 22 of the '024 patent.

Claim 5 requires that the stimulus that is applied to release the oligonucleotides "is

selected from the group consisting of a biological stimulus, a chemical stimulus, a thermal stimulus, an electrical stimulus, a magnetic stimulus, and a photo stimulus." As discussed above, the [redacted] is the claimed stimulus in the accused products, and Dr. Butte explains that "[t]he [redacted] is both a biological stimulus and a chemical stimulus." CX-0004C at Q/A 215.

Claim 17 requires that the claimed droplet "comprises a plurality of target nucleic acid analytes" and that "each of said plurality of target nucleic acid analytes attaches to an individual oligonucleotide molecule." As discussed above, the ddSEQ v1, [redacted] comprise a plurality of mRNAs, which attach to individual oligonucleotide molecules through hybridization and reverse transcription. *See* CX-0004C (Butte DWS) at Q/A 219-20. The droplets in the scATAC-seq [redacted] comprise a plurality of genomic DNA fragments, which attach to individual oligonucleotide molecules through hybdridization involving the Nextera Adaptor binding sequence. *Id.*

These processes also infringe the limitations of claim 19, which requires that "said given oligonucleotide molecule from said oligonucleotide molecules attaches to said target nucleic acid analyte by hybridization." *See* CX-0004C (Butte DWS) at Q/A 223.

There is no dispute that the ddSEQ system infringes the limitations of claim 22, which requires that the "porous gel bead comprises a polymer gel" and "said polymer gel is a polyacrylamide." *See* CX-0004C (Butte DWS) at Q/A 224-26.

Accordingly, the accused ddSEQ v1 [redacted] infringe dependent claims 5, 17, 19, and 22 of the '024 patent.

3. Indirect Infringement

The asserted claims of the '024 patent are method claims, and 10X contends that there is

a violation of section 337 by Bio-Rad based on theories of contributory and induced infringement. CIB at 30-37.

a. Underlying Direct Infringement

Indirect infringement requires evidence of an underlying direct infringement. As discussed above, ordinary use of the ddSEQ products would be direct infringement of the asserted claims of the '024 patent by Bio-Rad's customers. *See* CIB at 31-32. There is no dispute that Bio-Rad's customers have used and continue to use the ddSEQ v1 products in the United States. *Id.*; SIB at 36. In particular, 10X cites evidence that by early 2017, Bio-Rad had engaged with [redacted] of ddSEQ v1 products. CX-0004C (Butte DWS) at Q/A 599; CX-1494C; CX-1584C. Dr. Kaihara testified at her deposition that she has helped many Bio-Rad customers use the ddSEQ v1 system, including several in the United States. CX-0016C at 48-49; *see* Tr. 270. Bio-Rad's corporate representatives confirmed that Bio-Rad had sold [redacted] to its customers. CX-0019C (Reifsnyder Dep. Tr.) at 70-71; CX-0020C (Norton Dep. Tr.) at 32-33. This evidence is sufficient to show direct infringement of the '024 patent by Bio-Rad's customers.

[redacted] 10X contends that

[redacted]

Dr. Kaihara's testimony is sufficient to show direct infringement by [redacted]

b. Induced Infringement

Bio-Rad admits that it had knowledge of the application for the '024 patent [redacted], CX-0050C at 8, and the complaint in this investigation explicitly accused Bio-Rad of induced infringement. Complaint ¶ 72; *see Certain Television Sets, Television Receivers, Television Tuners, and Components Thereof*, Inv. No. 337-TA-910, Comm'n Op. at 39-43 (Oct. 14, 2015) (holding that service of the complaint on a respondent is sufficient to establish knowledge for indirect infringement). 10X identifies Bio-Rad's promotional materials and instruction manuals as evidence that Bio-Rad has induced infringement of the asserted claims. CIB at 36-37. This includes advertising materials, instructional manuals, and materials describing Bio-Rad's customer support and services for installation, repair, and troubleshooting of ddSEQ products. *See* CX-0004C (Butte DWS) at Q/A 629-37. Bio-Rad does not dispute this evidence of inducement, and Staff agrees with 10X that the dissemination of these materials is sufficient to show that Bio-Rad has induced infringement of the asserted claims of the '024 patent by the ddSEQ v1 products. SIB at 39-40.

Accordingly, 10X has shown that Bio-Rad has induced infringement of claims 1, 5, 17, 19, and 22 of the '024 patent by the ddSEQ v1 products.

c. Contributory Infringement

As discussed above, Bio-Rad had knowledge of its contributory infringement upon service of the complaint in this investigation. *See* Complaint ¶ 73 (alleging contributory infringement). Dr. Butte explained how the accused components of Bio-Rad's ddSEQ system are especially adapted for use in practicing the infringing methods. CX-0004C at Q/A 602-609. With respect to the ddSEQ v1 products, Dr. Butte identifies specific components, including the ddSEQ cartridges, ddSEQ single-cell isolator, ddSEQ cartridge holder, and consumables and assays used with the ddSEQ v1 process, including the SureCell WTA 3' v1 assay, which are designed and adapted for performing the infringing ddSEQ v1 workflow. *Id.* at Q/A 604. These components and their use in the ddSEQ v1 system are described in Bio-Rad product literature, including a [redacted] presentation (JX-0088C), and numerous instruction manuals and training materials. *See, e.g.,* CX-1405C; CX-1406C; CX-1435C; CX-1436C; CX-1460C; CX-1437C; CX-1451C; CX-1452C; CX-1454C; CX-1461C; CX-1473C; CX-1488C. Dr. Butte also identifies [redacted]. *Id.* at Q/A 605-609.

Bio-Rad disputes 10X's allegations of contributory infringement by arguing that the ddSEQ v1 system has a substantial non-infringing use. RIB at 66-68. Specifically, Dr. Metzker describes the Drop-seq protocol, where the barcode molecules are not releasably attached to the gel bead and are not released, as required by the claims of the '024 patent. RX-0665C at Q/A 138-143. During the course of this investigation, Bio-Rad developed a Drop-seq protocol for its ddSEQ system, releasing the protocol to the public in late 2018. *Id.* at Q/A 144-147; JX-0131C; JX-0130; *see* Tr. (Kaihara) at 239.

10X does not dispute that Drop-seq would be a non-infringing use of the ddSEQ system, but 10X and Staff argue that it is not a "substantial non-infringing use," because there is no evidence that any ddSEQ user has actually used the Drop-seq protocol. CIB at 34-35; SIB at 37-38. The Bio-Rad employee who was responsible for updating the Drop-seq protocol admitted at the hearing that she was not aware of any customer using Drop-seq on any Bio-Rad ddSEQ device. Tr. (Kaihara) at 240-41. Bio-Rad did not publish the Drop-seq protocol for ddSEQ until after the close of discovery in this investigation. *Id.* at 239. Dr. Butte considered the evidence regarding Bio-Rad's Drop-seq protocol and offered his opinion that it would not be a substantial use of the ddSEQ products because it would require additional reagents not included in the ddSEQ products, and it would not use several of the accused ddSEQ components, including Bio-Rad's SureCell kits and certain assays. CX-0004C at Q/A 611-616. Based on this evidence, I agree with 10X and Staff that the Drop-seq protocol is not a substantial non-infringing use of the ddSEQ system, and accordingly, 10X has carried its burden to show contributory infringement with respect to the accused ddSEQ v1 products. *See Certain Beverage Brewing Capsules, Components Thereof, and Products Containing the Same*, Inv. No. 337-TA-929, Comm'n Op. at 22-24 (Apr. 5, 2016) (finding contributory infringement based on a lack of substantial non-infringing uses).

Accordingly, 10X has shown that Bio-Rad contributorily infringed of claims 1, 5, 17, 19, and 22 of the '024 patent by importing and selling components of the ddSEQ v1 system.

D. Domestic Industry

There is no dispute that 10X's DI products practice claims 1, 5, 17, 19, and 22 of the '024 patent. CIB at 37-40; SIB at 40-41. 10X relies on the testimony of Dr. Butte to show that the DI products practice the asserted claims. CX-0004C at Q/A 227-287.

1. Claim 1

10X's DI products are part of a method of sample preparation of gDNA or mRNA for sequencing applications. CX-0004C (Butte DWS) at Q/A 256-58. The steps in the method meet each of the limitations of claim 1 of the '024 patent. *Id.* at Q/A 259-79. In particular, 10X's DI products provide a droplet that contains a porous gel bead formed of polyacrylamide. *Id.* at Q/A 260-61. 10X's single cell applications contain an mRNA as a target nucleic acid analyte, while the linked read solutions contain a gDNA fragment. *Id.* In each of the DI products, there are at least 1,000,000 oligonucleotide molecules that include barcode sequences. *Id.* at Q/A 263-64. These barcode sequences are the same for the oligonucleotide molecules on each gel bead. *Id.* at Q/A 269-70. The oligonucleotide molecules are releasably attached to the gel bead through a [redacted] that can be broken upon application of [redacted]. *Id.* at Q/A 265-68. [redacted] is applied to the gel bead as part of "Additive A," which cleaves the [redacted] to release the barcodes and also dissolves the gel bead. *Id.* at Q/A 272-73. Upon release, the barcodes attach through hybridization to the mRNA or gDNA fragment. *Id.* at Q/A 274-76. In the single-cell applications, a reverse transcription process then generates barcoded cDNA strands, which undergo further PCR outside the droplet to create barcoded double-stranded cDNAs. *Id.* at Q/A 278. In the linked-read applications, an isothermal amplification in the droplet creates a DNA amplicon, which undergoes further amplification outside the droplet. *Id.* at Q/A 279.

Accordingly, the DI products meet the technical prong of the domestic industry requirement with respect to claim 1 of the '024 patent.

2. Dependent Claims

The additional limitations of the asserted dependent claims are also practiced by the DI products. With respect to claim 5, [redacted]. CX-0004C (Butte DWS) at Q/A

281. With respect to claim 17, the mRNA of the single-cell applications and the gDNA of the linked-read applications are the target nucleic acid analytes, and they attach to individual oligonucleotide molecules in each of the DI products. *Id.* at Q/A 283. With respect to claim 19, the oligonucleotides attach to the mRNA or gDNA fragment through hybridization. *See Id.* at Q/A 274-76. With respect to claim 22, the porous gel beads of the DI products are comprised of polyacrylamide. *Id.* at Q/A 287.

Accordingly, the DI products meet the technical prong of the domestic industry requirement with respect to dependent claims 5, 17, 19, and 22 of the '024 patent.

E. Invalidity

Bio-Rad contends that the asserted claims of the '024 patent are invalid as anticipated or rendered obvious by U.S. Patent No. 9,347,059 (JX-0031, "the '059 patent") and/or U.S. Patent No. 9,902,950 (RX-0462, "Church"), alone or in combination with additional prior art. RIB at 68-111.

1. The '059 patent

The '059 patent issued from a patent application filed by Bio-Rad in April 2012, based on a provisional application that was filed in April 2011 by Dr. Saxonov, around the time that QuantaLife was acquired by Bio-Rad. CX-1829C (Saxonov RWS) at Q/A 25-27; JX-0031. Dr. Saxonov is the sole named inventor on the '059 patent, and Bio-Rad is the assignee. JX-0031. There is no dispute that the '059 patent is prior art to the '024 patent and all of the other asserted patents—it is listed as a cited reference on each of the asserted patents. *See* SIB at 41.

The '059 patent discloses methods for barcoding mRNA and DNA in droplets. The specification of the '059 patent explains the benefits of barcoding, allowing separately prepared samples to be pooled and sequenced, while "each sample can have its own unique barcode."

'059 patent, col. 3:53-62. The specification further describes "adaptors" with barcodes that "can be bundled within a partition, e.g., an aqueous phase of an emulsion, e.g. a droplet." *Id.*, col. 4:4-5. Also, "[t]he adaptor-filled droplets can be burst (e.g., through a temperature adjustment) to release reaction components . . . ." *Id.*, col. 4:34-37. The specification also describes "end modifications" that "can be attached to a nucleic acid strand through a linker." *Id.*, col. 12:30-36. Moreover, the specification describes "an amplification reaction" that "comprises a polymerase chain reaction." *Id.*, col. 2:41-46. The specification further provides that "[a] barcode can be attached to a polynucleotide by amplification with a primer comprising a barcode." *Id.* at col. 9:63-65.

There is no dispute that many of the limitations of the asserted claims of the '024 patent are disclosed in the '059 patent. In particular, the '059 patent discloses a method for sample preparation using a droplet containing barcoded oligonucleotide molecules and that the oligonucleotide molecules are subject to amplification. The parties dispute whether the '059 patent discloses several specific claim limitations, however, including the limitations regarding porous gel beads, and the limitations requiring releasable attachment to those beads.

a. Porous gel beads

10X and Staff argue that the '059 patent fails to disclose the porous gel beads claimed in the '024 patent. CIB at 62-75; SIB at 42-44. Bio-Rad concedes that there is no explicit disclosure of porous gel beads in the '059 patent specification. RRB at 20-21. Bio-Rad points to an embodiment described in the '059 patent where barcodes attach to a bead: "In some embodiments, antibodies can be linked to beads coated with short DNA fragments with a unique barcode." '059 patent, col. 36:59-60. Although the '059 patent does not describe the type of bead used with these antibodies, the use of the term "coated" suggests that the barcodes are

attached to the surface of a solid bead, rather than the interior of a porous bead. *See* CX-1872C (Dear RWS) at Q/A 108 ("Not only are the antibody-linked beads not being described as gel or porous, they are described as being 'coated' with short DNA fragments. Thus, the beads are not permeated with oligonucleotide molecules, and instead their surface is "coated." This further confirms that the beads are not porous gel beads, and are instead rigid, non-porous beads."); CX-1829C (Saxonov RWS) at Q/A 16 ("I was assuming that the beads were solid throughout or that only the exterior solid surface was going to be used."). Bio-Rad identifies an alleged reference to gel beads in another embodiment in the '059 patent specification describing a "next generation sequencing technique" called Roche 454 sequencing. '059 patent, col. 26:43-66. Dr. Metzker explains that the Roche 454 system used Sepharose beads, which he describes as porous gel beads. RX-0664C at Q/A 162-63, 166-68.

10X disputes Bio-Rad's assertion that the Roche 454 beads are porous gel beads as required by the claims of the '024 patent. CIB at 64-75. Although Dr. Metzker identifies the Roche 454 beads as Sepharose, there is no direct evidence in the record of the composition of these beads.[5] The only evidence that Bio-Rad cites is cross-examination testimony of 10X's expert, Dr. Dear, who identified a publication by Marcel Margulies *et al.* (CX-1940) describing the use of Sepharose beads in the context of Roche 454 sequencing: "Yes, I believe—at the time 454 published, I believe they used sepharose beads. That's the Margulies paper. Whether they did since in their commercial instruments, I don't know." Tr. 869-70.

10X also challenges Bio-Rad's assertion that Sepharose is a porous gel, relying on the opinion of Dr. Dear that Sepharose beads are rigid and lack the deformability that characterizes

[5] Certain evidence regarding Sepharose beads was excluded from Dr. Metzker's witness statement pursuant to a motion *in limine*. Order No. 38 at 8-9 (Mar. 12, 2019).

the gel beads described in the asserted patents. CIB at 64-75 (citing CX-1827C (Dear RWS) at Q/A 110-15). Bio-Rad supports its contention that Sepharose is a porous gel with testimony from Dr. Agresti, who describes Sepharose as "a crosslinked agarose, which is a material that I would consider to be a hydrogel," citing a document listing Sepharose as a "gel filtration media." RX-0503C (Agresti DWS) at Q/A 69; RX-0692. During cross-examination, however, Dr. Agresti admitted that gel filtration is different from microfluidics. Tr. 336. In addition, Dr. Agresti had previously testified at his deposition that he was not sure whether prior art using Sepharose would disclose a hydrogel bead. *Id.* at Q/A 66-68; Tr. 334.[6] Bio-Rad also offers testimony from Dr. Grenier describing Sepharose as a porous gel bead based on his work in graduate school in the mid-1990s. RX-0507C at Q/A 47-50.

Although I agree with Bio-Rad that Dr. Dear's strict requirements for rigidity and deformability may not be necessary to satisfy the "porous gel bead" limitation, Bio-Rad bears the burden on invalidity, and the conflicting evidence regarding Sepharose is neither clear nor convincing. Even if Bio-Rad had shown that Sepharose beads existed in the prior art that were porous gel beads, the record is far from clear that such beads were used in the Roche 454 sequencing process described in the '059 patent. Bio-Rad does not identify any disclosure in the '059 patent or the Margulies paper describing the composition or the characteristics of the Roche 454 beads, and Bio-Rad's witness testimony does not convincingly show that these beads described in the '059 patent are porous gel beads. Accordingly, Bio-Rad has failed to carry its

[6] Dr. Agresti equivocated on this issue when he was presented with a document filed at the USPTO by Bio-Rad's counsel when prosecuting a different patent application, which is discussed in more detail, *infra*, in the context of the Church patent. JX-0171.0027.

burden to show that the porous gel bead limitation of the '024 patent is anticipated by disclosures in the '059 patent.

b. Releasable attachment

Even if Bio-Rad had presented sufficient evidence at the hearing to show that the '059 patent disclosed porous gel beads, the beads identified by Dr. Metzker cannot anticipate the limitation of claim 1 of the '024 patent requiring that "said oligonucleotide molecules are releasably attached to said porous gel bead." 10X's expert, Dr. Dear, notes that the '059 patent describes the Roche 454 beads as "capture beads," and these beads are only discussed in the context of sequencing, which is a separate process in a separate embodiment from any discussion of releasing barcodes. CX-1827C at Q/A 87, 108. The '059 patent only references the Roche 454 beads as a substrate for sequencing, and Dr. Dear explains that "nucleic acids to be sequenced must remain attached to the substrate for their sequences to be determined." *Id.* at Q/A 108. Bio-Rad fails to connect the '059 patent's disclosure of Roche 454 beads to any discussion of releasable attachment, and the '059 patent's separate disclosure of these beads cannot form the basis for a finding of anticipation of this limitation. *See Net MoneyIN, Inc. v. VeriSign, Inc.*, 545 F.3d 1359, 1371 (Fed. Cir. 2008) (holding that the district court was "wrong to combine parts of the separate protocols shown in the iKP reference in concluding that claim 23 was anticipated").

Bio-Rad argues in the alternative that it would have been obvious to use porous gel beads for releasable attachment of the oligonucleotides described in the '059 patent. *See* RRB at 20-21. Dr. Metzker identifies the '059 patent's disclosure of antibody-linked beads and droplets as a disclosure of releasable attachment. RX-0664C at Q/A 181-85. In particular, the '059 patent specification describes an embodiment where "antibodies can be linked to beads coated with

short DNA fragments with a unique barcode," and further suggests that "[t]he antibodies could also be linked to droplets containing DNA fragments—which can be burst as appropriate." JX-0031, col. 36:59-64. Based on this disclosure, Dr. Metzker suggests that the '059 patent "teaches three interchangeable ways to deliver barcodes—droplets, capsules, and beads." RX-0664C at Q/A 181.

10X argues that Bio-Rad improperly mixes and matches different embodiments of the '059 patent. CIB at 53-64. The portion of the '059 specification that describes the release of "barcode adaptors" is limited to droplets, disclosing that "[t]he adaptor-filled droplets can be burst (e.g., through a temperature adjustment) to release reaction components (e.g., PCR or ligation components) that can be used for library preparation." JX-0031, col. 4:34-37. The antibody-linked beads are described in a separate embodiment, and the DNA fragments attached to these beads are not the barcodes described in the '059 patent's droplet embodiment. *See* CX-1827C (Dear RWS) at Q/A 40, 87. Moreover, although the '059 patent describes the droplets being "burst" to release barcode adaptors, there is no description of any mechanism for releasing the attached DNA fragments from beads. *Id.* at Q/A 155-56. Dr. Metzker concedes that the antibody-linked embodiment does not disclose the claimed barcodes but submits that "one of ordinary skill in the art would have immediately envisioned from the bead antibody disclosure in Saxonov that it could also apply to barcoding the cellular material." RX-0664C at Q/A 183. Dr. Metzker's suggestion that the antibody-linked DNA fragments could be replaced with barcodes is plausible, but this would only result in barcodes attached to beads, with no teaching regarding release.

To meet the releasable attachment limitation, Dr. Metzker further suggests that "[t]he only way for the barcodes in the inner droplet to function is by having them released from the

inner droplet," and "[o]ne of ordinary skill in the art would have therefore recognized that the barcodes on beads would be functioning in the same way, they would be released from the bead." *Id.* at Q/A 184. There is no disclosure in the '059 patent indicating how the beads could function to release barcodes in the same way as the burstable droplets, however, and Bio-Rad fails to offer a convincing argument for how one of ordinary skill in the art would use prior art teachings to replace the burstable droplets with beads. As Dr. Dear explains, the '059 patent specification shows that "although Saxonov specifically had the idea of releasing adaptors from a droplet," he "did not have the idea of releasing short DNA fragments from a bead." CX-1827C at Q/A 161.

Dr. Metzker attempts to supply a mechanism for releasably attaching barcodes to beads by suggesting that one of ordinary skill in the art would recognize that certain parts of the barcodes disclosed in the '059 patent "would be susceptible to cleavage and could remove the barcode adaptor molecule at the point of contact with the bead." RX-0664C at Q/A 186. This conclusory expert opinion cannot meet Bio-Rad's burden on invalidity, however. *See K/S Himpp v. Hear-Wear Techs., LLC*, 751 F.3d 1362, 1365-66 (Fed. Cir. 2014) (affirming a finding of non-obviousness where the USPTO properly rejected "a conclusory assertion from a third party about general knowledge in the art without evidence on the record," noting that the limitation "an important structural limitation that is not evidently and indisputably within the common knowledge of those skilled in the art!"). Dr. Metzker's suggestion for barcode cleavage is not based in any prior art disclosure but on hindsight, using the limitations of the '024 patent to selectively modify the prior art. *See Ortho-McNeil Pharm., Inc. v. Mylan Labs., Inc.*, 520 F.3d 1358, 1364 (Fed. Cir. 2008) ("In other words, Mylan's expert, Dr. Anderson, simply retraced the path of the inventor with hindsight, discounted the number and complexity of the alternatives,

and concluded that the invention [] was obvious. Of course, this reasoning is always inappropriate for an obviousness test . . . .").

Accordingly, I agree with 10X and Staff that Bio-Rad has failed to show that releasably attaching oligonucleotide molecules to a bead would be obvious in view of the '059 patent.

c. Combinations with other references

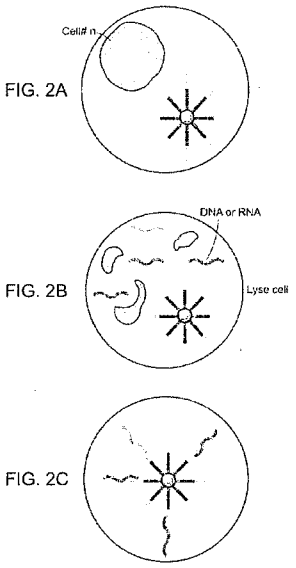
Bio-Rad further contends that the claimed porous gel beads are disclosed in other prior art references that would have been obvious to combine with the '059 patent. RIB at 77-80. These references include the Church patent (RX-0462), an article by Dr. Adam Abate, *Beating Poisson Encapsulation Statistics Using Close-Packed Ordering* (RX-0102, "Abate"), and U.S. Patent Application Pub. No. 2010/0304982, naming inventors Wolfgang Hinz *et al.* (RX-0461, "Hinz"). These references each disclose beads that appear to meet the "porous gel bead" limitations of the asserted claims of the '024 patent, but Bio-Rad fails to offer any credible motivation for combining the gel beads disclosed in these references with the droplet-based barcoding system disclosed in the '059 patent. As discussed above, Dr. Metzker's proposal for replacing the '059 patent's burstable droplets with beads having releasably attached barcodes is conclusory and relies on hindsight. Bio-Rad has identified no credible motivation for one of ordinary skill in the art to look to the gel beads disclosed in Church, Abate, or Hinz for releasable attachment of the barcodes contained in droplets in the '059 patent. Accordingly, Bio-Rad has failed to show that any asserted claim of the '024 patent is rendered obvious by the '059 patent in combination with these additional references.

2. The Church patent

The Church patent issued from a patent application filed in October 2011 and is assigned to Harvard College. RX-0462. There is no dispute that the Church patent is prior art to the '024

patent and all of the other asserted patents—the published patent application for the Church patent is listed as a cited reference on each of the asserted patents. *See* SIB at 50-51.

Church describes a process for producing beads coated with barcoded oligonucleotides. RX-0462, col. 2:28-34. The specification explicitly discloses "a variety of materials" for its beads, including "paramagnetic materials, ceramic, plastic, glass, polystyrene, methylstyrene, acrylic polymers, titanium, latex, sepharose, cellulose, nylon and the like." *Id.*, col. 12:38-42. Figure 2 of Church depicts a process where a single cell and barcoded bead are captured in an emulsion (Fig. 2A), nucleic acid sequences are released into the emulsion upon cell lysis (Fig. 2B), and the nucleic acid target is annealed to the barcoded bead (Fig. 2C).



Id. at Fig. 2, col. 3:49-53, col 5:50-6:10. The barcoded beads are then further processed, with cDNA synthesis for RNA, followed by PCR amplification. *Id.*, col. 6:18-24.

There is no dispute that the Church patent discloses a method for sample preparation using a barcoded bead with oligonucleotides attached that are subject to amplification. The parties dispute whether Church discloses several limitations of the asserted claims of the '024

patent, however, including whether the bead is a porous gel bead and whether the barcoded oligonucleotides are releasably attached.

a. Porous gel beads

Bio-Rad primarily relies on the Church patent's disclosure of Sepharose beads to anticipate the porous gel bead limitation of the '024 patent. RIB at 95-96. As discussed above in the context of the '059 patent, Bio-Rad relies on the testimony of Dr. Metzker and certain other evidence that Sepharose beads are porous gel beads. RX-0664C (Metzker DWS) at Q/A 213; *see also* RX-0503C (Agresti DWS) at Q/A 66-69; RX-0507C (Grenier DWS) at Q/A 47-50. 10X's expert, Dr. Dear, disagrees with Dr. Metzker's opinion, contending that Sepharose beads are rigid and lack the deformability to meet the gel bead limitation. CX-1827C (Dear RWS) at Q/A 110-115. Moreover, Bio-Rad's counsel represented to the USPTO in August 2017 that "Church does not teach or suggest particles that are hydrogels nor cleaving the oligonucleotides from the particles as recited in the claims." JX-0171.0027, Applicant's Response to Final Office Action at 8 (Aug. 21, 2017).[7] On this record, Bio-Rad has failed to carry its clear and convincing burden to show that Church's disclosure of Sepharose as a bead material anticipates the claim limitation requiring a porous gel bead.

Bio-Rad further contends that Church's disclosure of cellulose and polystyrene as bead materials anticipates the porous gel bead limitation. RIB at 95-96. Bio-Rad offers little evidence to support these assertions, however. With respect to cellulose, Bio-Rad cites cross-examination testimony from Dr. Dear, where he was presented with a catalog describing cellulose as having

[7] Bio-Rad later filed a correction with USPTO withdrawing this statement, conforming their prosecution filings to their arguments here that "Sepharose is a cross-linked agarose with a porous structure and is a hydrogel." RX-0660 at 9.

porosity, but Dr. Dear testifies that "I'm familiar with cellulose in the ordinary sense of the word, and as I know it, it's – as I have encountered it, it's nonporous and rigid." Tr. 890-91. For polystyrene, Bio-Rad cites testimony from Dr. Metzker that "[p]olystyrene can be cross-linked" and that "there are only two choices . . . of going with either a nonporous or a porous polystyrene bead." Tr. 676-77. This expert testimony, without additional evidentiary support, is insufficient to meet Bio-Rad's clear and convincing burden, particularly when considered in the context of Bio-Rad's prior representation to the USPTO that "Church does not teach or suggest particles that are hydrogels." JX-0171.0027.

Accordingly, the porous gel bead limitations of the '024 patent are not anticipated by the Church patent.

b. Releasable attachment

With respect to the releasable attachment limitations of the '024 patent, Bio-Rad points to a paragraph in the Church patent's specification describing "functional groups attached to [a bead] surface, which can be used to bind one or more reagents described herein to the bead." RX-0462, col. 12:43-53. Church further states: "One or more reagents can be attached to a support (e.g., a bead) by hybridization, covalent attachment, magnetic attachment, affinity attachment and the like." *Id.* Church then references "a variety of attachments" that "are commercially available" and states that beads "may also be functionalized using, for example, solid-phase chemistries known in the art," citing another patent, U.S. Patent No. 5,919,523 to Sundberg, *et al.* (RX-0466, "Sundberg"). *Id.*

According to Dr. Metzker, Sundberg "teaches the attachment of oligonucleotides to porous gel bead surfaces for the synthesis of oligonucleotides using spacer molecules." RX-0664C at Q/A 222. Sundberg provides that "[i]n some embodiments, the spacer may provide for

a cleavable function by way of, for example, exposure to acid or base." RX-0466, col. 8:57-59. In addition, "[a]ccording to other embodiments, small beads may be provided on the surface, and compounds synthesized thereon may be released upon completion of the synthesis. *Id.*, col. 6:14-16. Based on these disclosures, Bio-Rad argues that the releasable attachment limitation of the '024 patent is rendered obvious by Church, incorporating Sundberg by reference. RIB at 100-102; RRB at 30-34.[8]

10X and Staff disagree with Bio-Rad's contention, arguing that Sundberg is only cited by Church in the context of attaching functional groups to bind reagents to beads, without any discussion of releasing barcodes. CIB at 95-96; SRB at 19-20; *see* CX-1927C (Dear RWS) at Q/A 286. Moreover, the disclosures in Sundberg relied upon by Dr. Metzker are found under the heading "Pin-Based Methods," which is separate from "Bead Based Methods." *See* RX-0466, col. 8:23-59, 8:60-13:49. As Dr. Dear explains, the pin-based methods in Sundberg are methods of synthesis where "[e]ach tray is filled with a particular reagent for coupling in a particular chemical reaction on an individual pin." CX-1827C at Q/A 289 (quoting RX-0466, col. 8:33-34). According to Dr. Dear, the "cleavable function" cited by Dr. Metzker relates to the removal of a substance synthesized on a pin, not the release of barcodes attached to a bead. *Id.* Dr. Dear also notes that where Sundberg references release in the context of beads, it describes release "upon completion of the synthesis," which is not a release of barcodes into a droplet, as claimed

[8] It is unclear from Bio-Rad's post-hearing briefs whether it contends that this limitation is anticipated by the Church patent. Bio-Rad's initial post-hearing brief contains a section heading stating that "Church anticipates or renders obvious the claims of the '024 Patent." RIB at 94; *see also id.* at 106. Nevertheless, Bio-Rad does not appear to make an explicit contention that Church anticipates the "releasably attached" limitation of the '024 patent, *see* RIB at 100-102, and in Bio-Rad's post-hearing reply brief, Bio-Rad only contends that Church renders the asserted claims obvious. RRB at 30-34.

in the '024 patent. *Id.* Moreover, there is nothing in Church to suggest that one of ordinary skill would look to Sundberg for methods of releasing barcodes, because Church only describes a process where the barcodes remain attached to the bead. *Id.* These are significant gaps in Dr. Metzker's analysis that undercut Bio-Rad's obviousness contentions.

Bio-Rad's contentions are further contradicted by the representation of its attorneys to the USPTO that "Church does not teach or suggest particles that are hydrogels nor cleaving the oligonucleotides from the particles as recited in the claims." JX-0171.0027, Applicant's Response to Final Office Action at 8 (Aug. 21, 2017).[9] On this record, I agree with 10X and Staff that Bio-Rad has failed to show that the "releaseably attached" limitation is obvious in view of Church and Sundberg.

c. Combination with other references

Bio-Rad further contends that the asserted claims of the '024 patent are obvious in view of Church in combination with several additional references. RIB at 94-111; RRB at 30-34.

I agree with Bio-Rad that the use of a porous gel bead would have been obvious in view of Church in combination with Sundberg or Hinz (RX-0461). The list of bead materials in Church is non-exhaustive. RX-0462, col. 12:38-42 ("Beads may comprise a variety of materials including, but not limited to paramagnetic materials, ceramic, plastic, glass, polystyrene, methylstyrene, acrylic polymers, titanium, latex, sepharose, cellulose, nylon and the like."). Sundberg explicitly describes "polymer-coated supports" including "polyacrylamides." RX-0466, col. 5:32-38. Hinz teaches using polyacrylamide gel beads for nucleic acid analysis,

[9] Although Bio-Rad later retracted its argument regarding hydrogels, *see* n.7, *supra*, there has been no retraction of its statement reading cleaving the oligonucleotides. Bio-Rad submits that it amended the pending claims, however, to remove a limitation regarding cleaving the oligonucleotides. RRB at 33 (citing RX-0660.0005).

noting that these porous gel beads "allow polynucleotides to be attached throughout their volumes for higher loading capacities than those achievable solely with surface attachment." RX-0461, Abstract. Polyacrylamide is explicitly identified in the '024 patent specification and claims as a polymer gel that can be used for a porous gel bead. '024 patent, col. 1:51-53, 2:28-31. In addition, Dr. Dear agreed that Hinz discusses porous gel beads. Tr. 895-96. Accordingly, I agree with Bio-Rad that one of ordinary skill in the art, reading the statement in Church identifying a variety of bead materials, would have pursued other known materials available in the prior art, including the polyacrylamide beads described in Sundberg and Hinz. As recognized by Dr. Metzker, one of ordinary skill in the art would have been motivated to use these porous gel beads because of their increased loading capacities, consistent with the Church patent's stated goals of generating millions of barcoded beads for high-throughput sequencing. *See* RX-0664C at Q/A 216; RX-0462, col. 2:30-34, 2:51-3:6.

Bio-Rad has failed to make its case for obviousness with respect to the "releasably attached" limitation, however. Bio-Rad contends that it would have been obvious to use releasable attachments to the beads in Church when viewed in combination with the '059 patent, Sundberg, and the knowledge of one of ordinary skill in the art. RIB at 100-102; RRB at 30-34. Bio-Rad identifies no motivation for adding releasability of barcodes to the process disclosed in Church, however. As discussed above, Bio-Rad's attorneys argued to the USPTO that "Church does not teach or suggest . . . cleaving the oligonucleotides from the particles as recited in the claims." JX-0171.0027. Bio-Rad fails to identify any evidence in Church to contradict this prior representation.

Bio-Rad argues that the releasably attached limitation is obvious because there are only two options for the barcodes attached to the beads in Church: either the barcode remains attached

to the bead or it is detached. RRB at 32-33. Bio-Rad's argument relies on a misreading of *KSR*, however, which describes a case for obviousness where there is "a design need or market pressure to solve a problem," and one of ordinary skill would have "good reason to pursue the known options within his or her technical grasp." 550 U.S. at 421. Whether or not to release barcodes attached to bead does not present a choice of two solutions to a known problem, however—these are two methods for addressing different problems in the prior art. In Church, the problem is attaching barcodes to a bead, and these barcodes remain attached to the bead for synthesis and amplification. RX-0462, col. 6:18-24. In the '059 patent, droplets are burst to release reaction components with the barcodes. JX-0031, col. 4:34-36. Bio-Rad's framing of the issue as two known options has been constructed in hindsight, and it does not prove that this limitation is obvious. *See* CRB at 37-38.[10]

Accordingly, Bio-Rad has failed to show that any asserted claim of the '024 patent is rendered obvious by Church in combination with any of these additional references.

3. Secondary considerations of non-obviousness

In *Graham v. John Deere Co. of Kansas City*, the Supreme Court held that in determining obviousness "[s]uch secondary considerations as commercial success, long felt but unsolved needs, failure of others, *etc.*, might be utilized" as "*indicia* of obviousness or nonobviousness," 383 U.S. 1, 17-18 (1966). Indeed, "evidence of secondary considerations may often be the most probative and cogent evidence in the record." *In re Cyclobenzaprine Hydrochloride Extended-*

[10] At the hearing, Dr. Dear testified: "No, there's not only two options as to what you do. If you say do—I mean, there are many things you can do in droplets. If you simply say do we cleave it off the bead or do we not cleave it off the bead, the point I'm making is that that doesn't constitute a conception. It's just saying those are two options for that particular feature." Tr. 912:3-13.

Release Capsule Patent Litigation, 676 F.3d 1063, 1075-76 (Fed. Cir. 2012) (quoting *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 1538–39 (Fed. Cir. 1983)) (internal quotation marks omitted). Accordingly, such evidence "must always when present be considered *en route* to a determination of obviousness." *Id.* (quoting *Stratoflex,* 713 F.2d at 1538–39) (internal quotation marks omitted). Secondary considerations of non-obviousness "include: commercial success enjoyed by devices practicing the patented invention, industry praise for the patented invention, copying by others, and the existence of a long-felt but unsatisfied need for the invention." *Apple Inc. v. Samsung Electronics Co.*, 839 F.3d 1034, 1052 (Fed. Cir. 2016).

10X identifies five secondary considerations that it alleges weigh against a finding of obviousness. Four of these considerations—(1) solving a long-felt need, (2) industry praise, (3) commercial success, and (4) failure of others—relate to the success of 10X's domestic industry products and the failure of a competitor to develop a competing product. With regard to these secondary considerations, Bio-Rad argues that 10X has not shown a nexus between the asserted claims and the domestic industry products. RRB at 94-95. With respect to the fifth secondary consideration identified by 10X—copying by another, *viz.,* Bio-Rad—Bio-Rad contests 10X's allegations of copying.

a. The success of the domestic industry products weighs against obviousness.

i. 10X has established the required nexus.

There must be a "nexus between the merits of the claimed invention and evidence of secondary considerations . . . in order for the evidence to be given substantial weight in an obviousness decision." *Ruiz v. A.B. Chance Co.*, 234 F.3d 654, 668 (Fed. Cir. 2000)); *see also Ormco Corp v. Align Tech., Inc.*, 463 F.3d 1299, 1311-12 (Fed. Cir. 2006) ("Evidence of commercial success, or other secondary considerations, is only significant if there is a nexus

between the claimed invention and the commercial success."). As discussed herein, the domestic industry single-cell products practice all of the asserted patents, while the domestic industry linked-read products practice the asserted claims of the '024, '468, and '204 patents. Citing *WBIP, LLC v. Kohler Co.*, both 10X and Staff argue that the domestic industry products' practice of the asserted claims triggers a presumption that there is a nexus between the claims and the "asserted objective evidence" tied to the domestic industry products. 829 F.3d 1317, 1329 (Fed. Cir. 2016) (internal citations and quotation marks omitted). Such a presumption is only applicable, however, if a product is coextensive with the claimed invention. *Polaris Indus., Inc. v. Artic Cat, Inc.*, 882 F.3d 1056, 1072 (Fed. Cir. 2018) ("[W]hen the thing that is commercially successful is not coextensive with the patented invention—for example, if the patented invention is only a component of a commercially successful machine or process—the patentee must show prima facie a legally sufficient relationship between that which is patented and that which is sold.") (internal citation and quotation marks omitted). Neither 10X nor Staff provide analysis regarding whether the domestic industry products are coextensive with the claimed invention.

At least in some instances, the claimed invention is only a component of the domestic industry products. For example, the asserted claims of the '204 patent are directed to droplets containing capsules, wherein the capsules contain barcode molecules. *See, e.g.,* '204 patent, col. 44:42-49 (unasserted claim 1), col. 46:24-27 (claim 27). Although the domestic industry products have such capsules in the form of a gel beads, they also include unclaimed components, such as "microfluidic chips, chip holders, droplet generating instruments, . . . and various other reagents." CIB at 5. Accordingly, I find that the domestic industry products' practice of the asserted claims does not trigger the presumption of a nexus.

Although the presumption of a nexus does not apply, 10X has shown that the evidence relating to the success of its products is sufficiently related to the claimed invention to provide the necessary nexus. All of the asserted claims require a droplet containing a capsule that is capable of releasing barcode molecules. *See, e.g.,* CX-1827C (Dear RWS) at Q/A 966. This claimed invention is employed in the domestic industry products in the form of "GemCode" or "GEM technology," wherein gel beads capable of releasing barcode molecules are encapsulated in droplets. *Id.* at Q/A 966. Using GEM technology, the domestic industry products are able to achieve "high-throughput profiling of large numbers of single cells or molecules in a single procedure." *Id.* As discussed below, the ability to achieve a high-throughput was the key to the domestic products' success. Conversely, the failure of a competitor with a commanding position in the market to develop a high-throughput solution led to the competitor abandoning the market.

Bio-Rad counters that high throughput "is not a patented feature of the commercial product." RIB at 219. The domestic industry products, however, are only able to achieve a high throughput by using the claimed invention, *viz.*, by encapsulating gel beads with attached barcode molecules into droplets. CX-1827C (Dear RWS) at Q/A 967. This relationship between the domestic industry product's high throughput and the claimed invention provides the necessary nexus. *See Rambus Inc. v Rea,* 731 F.3d 1248, 1256-57 (Fed. Cir. 2013) (finding a nexus between evidence relating to the unclaimed high speed achieved by a memory system and the challenged claims, because the high speed was enabled by the claimed functionality).

ii. The success of the domestic industry products and the failure of a competitor to develop a competing product weigh against obviousness.

Bio-Rad does not dispute that the domestic industry products (1) solved a long-felt need, (2) received industry praise, (3) were a commercial success, and (4) that others failed in

developing a high-throughput system. With regard to long-felt need, at the time of the invention, it was widely recognized that it would be beneficial for both single-cell analysis and linked-read analysis to increase the single-run throughput of the single cells or molecules being analyzed. For instance, in the context of single-cell analysis, [redacted] *Id.*

At the time of the presentation, the industry leader in the field of sample preparation systems for SCG was Fluidigm. *Id.* at .00011; CX-0016C (Kaihara Dep. Tr.) at 25:19-26:3. In 2012, Fluidigm released its "C_1 Single-Cell AutoPrep System for cell isolation, sample prep, and analysis." CX-1946C.00011 (bold-face type removed), *see also, id.* at .00003 ("2012: Introduction of Fluidigm's SC automated cell prep instrument"). The Fluidigm system had throughput of "up to 96 cells per run." *Id.* at .00013. This, however, was inadequate, as it was necessary to analyze "many more single cells . . . within a single experiment" in order "to address biological and stoichiometric noise or at least to achieve a better understanding of cell-to-cell variation within tissue[.]" CX-1269.00005 (quoting Jokim Lundeberg, KTH Royal Institute of Technology (Sweden)) (internal quotation marks omitted). This left an [redacted]. In the 2014 timeframe, such a need was even acknowledged by Fluidigm's CEO: "As the science of single-cell analysis unfolds, it's clear to us the field is evolving in several important ways.

[There is] an increasing need for higher throughput to enable large volume studies." CX-1946C.00012 (quoting Fluidigm CEO, Gajus Worthington) (internal quotation marks omitted and alterations in original). In the context of linked-read technology, high throughput was also a "long-felt but unresolved need in accessing long range DNA sequence information." CX-1827C (Dear RWS) at Q/A 997.

Upon its introduction, 10X's GEM technology received industry praise and recognition. In particular, Dr. Hindson presented 10X's GEM technology at the Advances in Genome Biology Technology conference ("AGBT"). CX-0001C (Hindson WS) at Q/A 143-48. After Dr. Hindson described the data that was obtained through the technology, the audience applauded. *Id.* at 149-50. Dr. Agresti, who attended the conference on behalf of Bio-Rad, congratulated Dr. Hindson on "10X's achievements and said something to the effect that he's real amazed and it's awesome what we've done with the technology." *Id.* at Q/A 152-53. As acknowledged by Dr. Tzonev, another Bio-Rad witness, 10X's presentation at the AGBT [redacted] CX-0023C (Tzonev Dep. Tr.) at 127:19-25.

The domestic industry products have been a commercial success. Between the second quarter of 2015 and the second quarter of 2018, the domestic industry products generated $159 million in revenues. CX-1827C (Dear RWS) at Q/A 1073; JX-0043C. 10X has sold the domestic industry products to over 550 customers, including the National Institutes of Health, University of California, Harvard University, Cornell University, California Institute of Technology, Dartmouth College, Duke University, Georgetown University, John Hopkins University, and the University of Georgia. CX-1827C (Dear RWS) at Q/A 1071-72; CX-1265C.

The success of 10X's domestic industry products stands in marked contrast to Fluidigm's failure to develop a competing high throughput product. Although it was "the sole player in the single cell market in 2009" and recognized by at least 2014 that there was "an increasing need for higher throughput to enable large volume studies," Fluidigm was ultimately unable to develop a high throughput solution. CX-1827C (Dear RWS) at Q/A 1078-1083; CX-1691C.00024; CX-1946C.00012 (quoting Fluidigm CEO, Gajus Worthington). In a May 4, 2017 earnings call, Fluidigm acknowledged that its "single-cell genomics business," which was "overwhelmingly [Fluidigm's] C1 product line, was down in the quarter by over 70% year-on-year." CX-1273.00003. One of the reasons for the decline, according to Fluidigm, was "the announcement of new competition." *Id.* As a result of the decline, Fluidigm announced that it would continue "to shift [its] primary business focus" away from the single cell market. *Id.*

In view of the foregoing, I find that the domestic industry products solved a long-felt, but unmet need, received industry praise, were commercially successful, and that another tried but failed to develop a solution to satisfy the unmet need. I further find that this evidence weighs against obviousness.

b. 10X has not established that Bio-Rad copied the claimed invention.

10X argues that Bio-Rad's copying of the claimed invention shows that the invention is not obvious. 10X's argument that Bio-Rad copied its invention, however, is unpersuasive. 10X publicly disclosed its GEM technology for the first time at the February 2015 AGBT conference, which was attended by Dr. Agresti and two other Bio-Rad employees. CX-0001C (Hindson WS) at Q/A 143; 151-53. [redacted]. Prior to joining Bio-Rad, Dr. Agresti worked at

Amyris, where he [redacted]. By at least 2009, as shown in a paper that he co-authored, Dr. Agresti was aware that a gel beads could be used to deliver DNA molecules to droplets. RX-0102.00001 ("[T]he gel particles can be functionalized with a variety of compounds, including fluorophores, DNA fragments, antibodies, and enzymes."); RX-0503C (Agresti DWS) at Q/A 40. The paper notes that gel particles "are useful substrates for chemical and biological applications" and "[t]he compliance of the particles prevents clogging of the channels" of microfluidic devices. RX-0102.00001.

[redacted]

[redacted] The presentation attached to the email is JX-0065C; JX-0067C is a higher quality copy of the presentation. *Id.* at Q/A 49.

[redacted]. JX-0067C.00014; RX-0503C (Agresti DWS) at Q/A 54.

Accordingly, there is clear documentary evidence, as well as Dr. Agresti's testimony, showing that, [redacted]

In support of its argument that Bio-Rad copied the claimed invention, 10X points to Dr. Agresti's trial testimony and an [redacted]

[redacted]—is an advantage discussed in the 2009 paper he co-authored, entitled "Beating Poisson encapsulation statistics using close-packed ordering." RX-0102.0001. ("We use compliant gel particles in these experiments. The compliance of the particles prevents clogging of the channels.") (footnotes omitted).

[redacted]. 10X's argument is unpersuasive.

[redacted]

Based on the foregoing, I find that 10X has not shown that Bio-Rad copied the claimed invention.

V. THE '468 PATENT

A. Asserted Claims

10X is asserting claims 1, 6, 7, 9, and 21 of the '468 patent. Claim 1 is independent and the remaining claims depend directly or indirectly from claim 1. Claim 1 recites:

> A method for droplet generation, comprising:
>
> (a) providing at least 1,000,000 oligonucleotide molecules comprising barcode sequences, wherein said barcode sequences are the same sequence for said at least 1,000,000 oligonucleotide molecules, wherein said at least 1,000,000 oligonucleotide molecules are releasably attached to a bead, wherein said bead is porous;
>
> (b) combining said at least 1,000,000 oligonucleotide molecules and a sample comprising a nucleic acid analyte each in an aqueous phase at a first junction of two or more channels of a microfluidic device to form an aqueous mixture comprising said at least 1,000,000 oligonucleotide molecules attached to said bead and said sample; and

(c) generating a droplet comprising said at least 1,000,000 oligonucleotide molecules attached to said bead and said sample comprising said nucleic acid analyte by contacting said aqueous mixture with an immiscible continuous phase at a second junction of two or more channels of said microfluidic device.

'468 patent (JX-0005), col. 33:56-col. 34:9.

Claims 6, 7, 9, and 21 depend directly from claim 1. Claim 6 requires that the bead be formed from a polyacrylamide. *Id.*, col. 34:25-26. Claim 7 requires that the bead be a gel bead. *Id.*, col. 34:27. Claim 9 requires that the "at least 1,000,000 oligonucleotide molecules" have a region that functions as a primer. *Id.*, col. 34:30-32. Claim 21 requires that after the generation of a droplet "a given oligonucleotide molecule of said at least 1,000,000 oligonucleotide molecules attaches to said nucleic acid analyte," before being "subjected to nucleic acid amplification to yield a barcoded nucleic acid analyte." *Id.*, col. 35:3-9.

B. Claim Construction

The parties agreed to construe "barcode" to mean a "label that may be attached to an analyte to convey identifying information about the analyte." Order No. 22 at 2. In the *Markman* order, "1,000,000 oligonucleotides comprising barcode sequences" was construed to mean "1,000,000 oligonucleotide molecules that include, but are not necessarily limited to, barcode sequences." *Id.* at 17-22. The term "releasably attached" was construed to mean "attached in a manner that allows the attached object to be released." *Id.* at 22-30. The term "amplification" was construed to mean "increasing the number of copies of the target sequence to be detected," including by reverse transcription. *Id.* at 31-45.

C. Infringement

10X accuses Bio-Rad of infringing claims 1, 6, 7, 9, and 21 of the '468 patent.

1. Claim 1

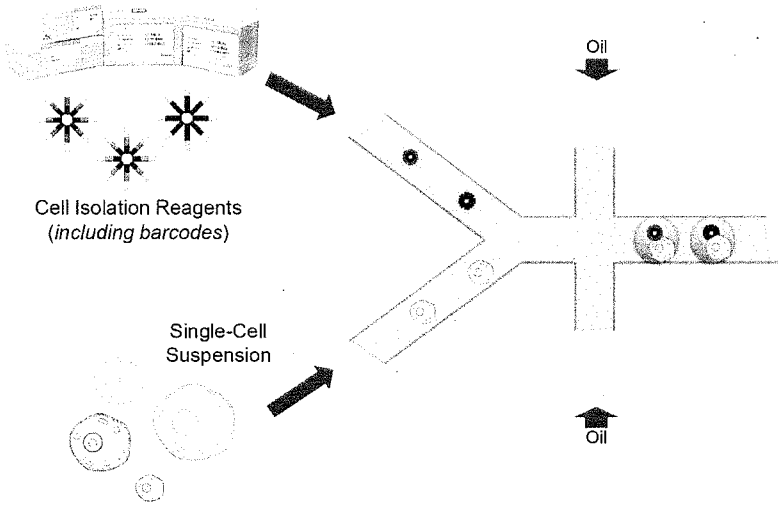
There is no dispute that Bio-Rad's ddSEQ system includes a method for droplet generation, and 10X relies on Dr. Butte's analysis to show infringement of the limitations of claim 1 of the '468 patent. CX-0004C at Q/A 418-437.

a. "providing at least 1,000,000 oligonucleotide molecules . . ."

There is no dispute with respect to a majority of the elements in the first limitation of claim 1 of the '468 patent, which includes limitations that are substantively identical to those discussed above for claim 1 of the '024 patent. Dr. Butte refers back to his analysis for the '024 patent for these limitations, which require "providing at least 1,000,000 oligonucleotide molecules," that the "barcode sequences are the same," that the molecules are "releasably attached to a bead," and "said bead is porous." CX-0004C at Q/A 422-424. As discussed above, Bio-Rad disputes infringement of the "releasably attached" limitation, but its non-infringement arguments are not consistent with the claim construction adopted in this investigation. Accordingly, the accused ddSEQ products infringe the "providing . . ." limitation of claim 1 of the '468 patent.

b. "combining said at least 1,000,000 oligonucleotide molecules and a sample comprising a nucleic acid analyte each in an aqueous phase . . ."

Dr. Butte identifies Bio-Rad documentation describing the mixing of two input aqueous solutions in the ddSEQ v1 process: one solution contains the oligonucleotide molecules and the other solution contains a sample of single cells comprising the mRNA nucleic acid analyte. CX-0004C at Q/A 427-28. Dr. Butte testifies that these two solutions are combined at a first junction of two channels of the ddSEQ v1 cartridge, citing a Bio-Rad document showing the mixing of the aqueous solutions. *Id.* at Q/A 429.



JX-0035.00009. He further cites the testimony of Bio-Rad employee Lucas Frenz, describing the junction where the two solutions are combined. CX-0011C (Frenz Dep. Tr.) at 229-30.

[redacted].

Bio-Rad contends that Dr. Butte's testimony fails to carry 10X's burden to show infringement of this limitation. RIB at 165-66.[12] In particular, Bio-Rad cites Dr. Butte's testimony on cross-examination where he agrees that [redacted] Tr. 408:6-13. Dr. Butte further testifies that "it would be a big mess" if the two solutions mixed "without forming a droplet." *Id.* at 409:12-21. As Dr. Butte further explains his testimony,

[12] Pursuant to Order No. 39 (Mar. 12, 2019), Bio-Rad was precluded from offering affirmative evidence of non-infringement regarding this limitation.

however, these statements do not contradict his infringement opinions. *See* SRB at 43-44. Dr. Butte explains that the two solutions [redacted] *Id.* And although he concedes that "lysis might start, it takes time to complete." *Id.* at 408:20-498:2. When Dr. Butte's cross-examination testimony is considered in the context of his infringement opinions and the evidence from Bio-Rad's documents and testimony, there is a preponderance of evidence that the ddSEQ system infringes the "combining" limitation.

c. "generating a droplet . . ."

There is no dispute with respect to the elements of the final limitation of claim 1. Dr. Butte identifies evidence that a droplet is formed when the mRNA or genomic DNA fragment contacts the aqueous mixture at a second junction. CX-0004C at Q/A 436-37. This droplet generation is depicted in Bio-Rad documents.

JX-0088C.00013. Dr. Butte explains how the limitation is met for both the ddSEQ v1 [redacted] products. CX-0004C at Q/A 436-37. Dr. Frenz confirmed the location of the junction in the ddSEQ v1 [redacted] cartridges. CX-0011C at 231-32; CX-0056C; CX-1458C.

Accordingly, both the ddSEQ v1 [redacted] processes infringe the method of claim 1 of the '468 patent.

2. Dependent Claims

There is no dispute with respect to the ddSEQ system's infringement of the limitations in dependent claims 6, 7, 9, and 21 of the '468 patent. CIB at 197-98; SIB at 92.

As discussed in the context of the '024 patent, there is no dispute that the ddSEQ system uses a gel bead comprised of [redacted], as required by claims 6 and 7 of the '468 patent. *See* CX-0004C (Butte DWS) at Q/A 224-26, 438-43.

Claim 9 of the '468 patent requires that the oligonucleotide molecule "comprises a region which functions as a primer." Dr. Butte explains that in the WTA 3' v1 [redacted]. CX-0004C at Q/A 446. In the scATAC-seq [redacted] assays, the Nextera Adaptor binding sequence attaches to the Nextera Adaptor and functions as a primer during PCR in the droplet. *Id.*

Claim 21 of the '468 patent requires that the "nucleic acid analyte is subjected to nucleic acid amplification to yield a barcoded nucleic acid analyte." The limitations of this claim are similar to those recited in limitation (c) of claim 1 of the '024 patent, and the ddSEQ system infringes claim 21 for the same reasons discussed above. *See* CX-0004C (Butte DWS) at Q/A 449.

Accordingly, [redacted] the ddSEQ v1 [redacted] processes infringe claims 6, 7, 9, and 21 of the '468 patent.

3. Indirect Infringement

10X accuses Bio-Rad of indirect infringement of the method claims of the '468 patent based on the same evidence cited for the '024 patent. CIB at 198. Staff and Bio-Rad raise the same indirect infringement arguments for the '468 patent that were addressed in the context of the '024 patent. RIB at 166; SIB at 92-93.

As discussed above, 10X has shown that the ddSEQ v1 system has been used in the United States, [redacted]. For the same reasons discussed above in the context of the '024 patent, 10X has thus shown that Bio-Rad has induced infringement and contributorily infringed claims 1, 6, 7, 9, and 21 of the '468 patent by importing and selling components of the ddSEQ v1 system.

D. Domestic Industry

10X contends that its DI products practice claims 1, 6, 7, 9, and 21 of the '468 patent, relying on the testimony of Dr. Butte. CIB at 198-201; CX-0004C at Q/A 450-77.

1. Claim 1

There is no dispute that the DI products include a method for droplet generation, and 10X relies on Dr. Butte's analysis to show infringement of the limitations of claim 1 of the '468 patent. CX-0004C at Q/A 453-66.

There is no dispute with respect to the first limitation of claim 1 of the '468 patent, which includes limitations that are substantively identical to those discussed above for claim 1 of the '024 patent. Dr. Butte refers back to his analysis for the '024 patent for these limitations, which require "providing at least 1,000,000 oligonucleotide molecules," that the "barcode sequences are the same," that the molecules are "releasably attached to a bead," and "said bead is porous." CX-0004C at Q/A 455-56.

With respect to the second limitation of claim 1 of the '468 patent requiring forming an aqueous mixture, Dr. Butte identifies two aqueous input solutions for 10X's single-cell products: one solution comprising an mRNA nucleic acid analyte and a second solution including gel beads with oligonucleotide molecules attached. CX-0004C at Q/A 458-59. He further identifies a junction of channels on the Chromium Single Cell 3' microfluidic chip where the two solutions

are combined. *Id.* at Q/A 460. With respect to 10X's linked-read products, Dr. Butte identifies a Sample Master Mix with denatured genomic DNAs and a second solution containing gel beads. *Id.* at Q/A 462. He further identifies the junction on the Chromium Genome microfluidic chip where the two solutions are combined. *Id.* at Q/A 463. Bio-Rad argues that 10X has failed to carry its burden with respect to the "aqueous solution" limitation, raising arguments similar to those discussed above in the context of infringement. RIB at 166-67. As discussed above, however, Dr. Butte's testimony on cross-examination does not contradict his affirmative opinions with respect to this limitation.

There is no dispute with respect to the third limitation of claim 1 of the '468 patent, which requires generating a droplet. Dr. Butte identifies images of the claimed second junction in the 10X single-cell and linked-read applications and documents showing the portioning oil loaded on the Genome microfluidic chip. CX-0004C at Q/A 465-66; CX-0581C; CX-0622C; CX-0481; CX-0578.

Accordingly, the DI products practice claim 1 of the '468 patent.

2. Dependent Claims

There are no disputes with respect to the limitations recited in dependent claims 6, 7, 9, 10, 17, and 21 of the '468 patent. CIB at 200-01; SIB at 94.

With respect to claims 6 and 7, Dr. Butte refers back to his opinions with respect to the '024 patent, and there is no dispute that the DI products use a polyacrylamide gel bead. CX-0004C at Q/A 467-70.

With respect to claims 9 and 10, Dr. Butte explains that the DI products use the 10X Barcoded Primer—in the single-cell applications, the poly-T sequence attaches to the mRNA and functions as a primer during reverse transcription, and in the linked-read applications, a 6

nucleotide random primer is used in isothermal amplification. CX-0004C at Q/A 472; CX-0579; CX-0578.

With respect to claim 17, Dr. Butte explains that the gel bead is dissolved upon application of [redacted]. CX-0004C at Q/A 476.

With respect to claim 21, Dr. Butte refers to his testimony regarding the '024 patent, explaining how the DI products undergo amplification. CX-0004C at Q/A 478.

Accordingly, the DI products practice claims 1, 6, 7, 9, 10, 17, and 21 of the '468 patent.

E. Invalidity

Bio-Rad contends that the asserted claims of the '468 patent are invalid as anticipated or rendered obvious by the '059 patent (JX-0031), alone or in combination with additional prior art, including Hinz (RX-0461), PCT Pub. No. WO 2010/036352 A1 naming inventors Billy W. Colston, Jr. and Benjamin J. Hindson, *et al.* (RX-0473, "Colston"), and U.S. Patent App. Pub. No. US 2012/0220494 A1 naming inventor Michael Samuels *et al.* (RX-0474, "Samuels"). RIB at 168-85.

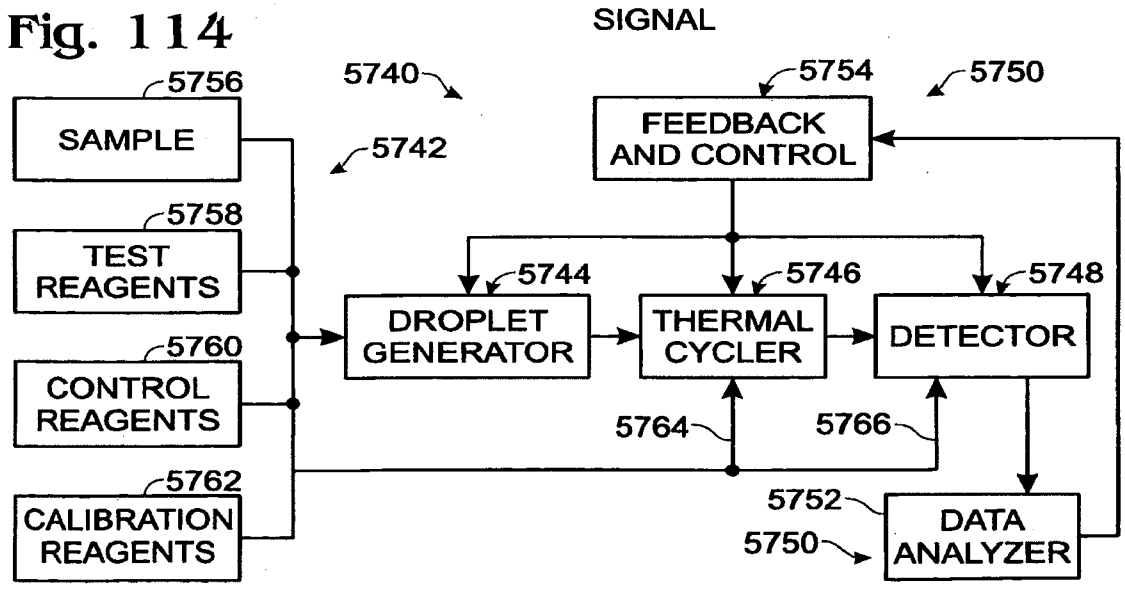
1. "at least 1,000,000 oligonucleotide molecules . . . releasably attached to a bead"

As discussed above in the context of the '024 patent, Bio-Rad has failed to show that the '059 patent anticipates or renders obvious, alone or in combination with other references, the claim limitation requiring that oligonucleotide molecules be releasably attached to a bead. For this reason alone, Bio-Rad has not shown that the asserted claims of the '468 patent are invalid.

2. "combining said at least 1,000,000 oligonucleotide molecules and a sample comprising a nucleic acid analyte each in an aqueous phase at a first junction"

Bio-Rad contends that the "combining" step of claim 1 of the '468 patent is anticipated or rendered obvious by the '059 patent through the incorporation by reference of Colston. RIB at

170-79. In the '059 patent's discussion of droplet generation, the specification states that droplets may be generated by devices described in Colston. JX-0031, col. 13:8-14. Colston is a patent application published in April 2010 that is assigned to QuantaLife, naming Dr. Hindson as one of the co-inventors. RX-0473. Colston teaches that "samples and/or reagents may be . . . mixed selectably before they are supplied to a downstream region of the system," identifying a "droplet generator" as one such region. *Id.* at 243. Figure 114 of Colston is a schematic showing the mixing of a sample and reagents prior to a droplet generator.



RX-0473.000340, Fig. 114. Based on these disclosures, Dr. Metzker submits that the '059 patent discloses the combination of a nucleic acid analyte sample and oligonucleotide molecules at a first junction to form an aqueous mixture. RX-0664C at Q/A 264.

10X contends that Bio-Rad's anticipation argument fails because the '059 patent only references Colston in the context of droplet generation, with no discussion of mixing polynucleotides and barcode adaptors. CIB at 201-02. Dr. Dear notes that the '059 patent describes the step of combining adaptors with polynucleotides as "merging," rather than droplet

formation. CX-1827C at Q/A 401 (citing JX-0031, col. 1:40-51). On this record, I agree with 10X that the "combining" limitation is not anticipated the '059 patent's incorporation of Colston by reference.

Bio-Rad further contends that it would have been obvious to combine the '059 patent's polynucleotides and barcode adaptors with the microfluidics disclosed in Colston. RIB at 170-72. Dr. Metzker suggests that one of ordinary skill "would have been motivated . . . to keep assay reagents separate from the nucleic acid or cellular analyte solutions" and "would have been motivated to see what methods others have used in droplet formation devices to improve the efficiency of her system." RX-0664C at Q/A 265. 10X disagrees with these obviousness contentions, arguing that Colston's disclosures are too vague to render obvious the "first junction" limitation of the '468 patent. CIB at 201-02. As explained by Dr. Dear, Colston does "not disclose how reagents and samples are combined, beads with barcodes, a junction of two channels to form an aqueous mixture of beads with barcodes and sample, and generation of droplets with beads and sample at a second junction." CX-1827C at Q/A 403. Dr. Dear further criticizes Dr. Metzker's reliance on Figure 114, because it is a "schematic" rather than a "microfluidic layout." *Id.* at Q/A 404. In reply, Bio-Rad argues that the '468 patent itself has no figures illustrating the claimed junctions and cites cross-examination testimony from Dr. Dear admitting that Colston shows mixing of the sample and reagents in an aqueous phase. RRB at 85; Tr. (Dear) at 902.

Although I agree with Bio-Rad that the disclosures in Colston are sufficient to show the mixing of a sample and reagents in an aqueous phase, Bio-Rad has failed to offer clear and convincing evidence that it would have been obvious to apply this mixing to the polynucleotides and barcode adaptors in the '059 patent. Dr. Metzker only offers conclusory opinions regarding

the motivations of one of ordinary skill in the art to implement a microfluidics system meeting the limitations of the '468 patent. RX-0664C at Q/A 265. Bio-Rad fails to cite any evidence from the '059 patent or other contemporaneous references indicating a need or desire to implement a particular microfluidic mixing process for the '059 patent's polynucleotides and barcode adaptors. Accordingly, Bio-Rad has failed to carry its burden to show that this limitation is obvious in view of the '059 patent, alone or in combination with Colston.

Bio-Rad's proposed combinations of the '059 patent with other references fail for the same reason, because Dr. Metzker only offers conclusory opinions regarding the obviousness of combining these references. *See* RX-0664C at Q/A 268. Although Samuels (RX-0474), Song (RX-0475), and Abate (RX-0102) teach microfluidic systems that meet at least some of the claim limitations of the '468 patent, Bio-Rad fails to identify a credible reason for implementing these processes to mix the polynucleotides and barcode adaptors of the '059 patent. There is no evidence that these references solve a known problem for the process described in the '059 patent, and there is no evidence that the particular microfluidic systems identified by Bio-Rad are among a finite number of identified, predictable solutions. Accordingly, Accordingly, Bio-Rad has failed to carry its burden on obviousness with respect to this limitation.

3. "generating a droplet . . . by contacting said aqueous mixture with an immiscible continuous phase at a second junction"

Bio-Rad contends that the "generating a droplet" step of claim 1 of the '468 patent is anticipated or rendered obvious by the '059 patent alone or in combination with Colston and Samuels. RIB at 179-83. In particular, Bio-Rad cites a disclosure in the '059 patent that "[m]icrofluidic methods of producing emulsion droplets using microchannel cross-flow focusing on physical agitation can produce either monodisperse or polydisperse emlusions." JX-0031, col. 14:6-8. Dr. Metzker submits that "[o]ne of ordinary skill in the art would have understood

that the 'cross-flow focusing' as taught by Saxonov involves the concept of flow-focusing using a cross junction with two or more input channels, one being oil and one being an aqueous channel." RX-0664C at Q/A 276.

The '059 patent's reference to "cross-flow focusing" is not sufficient to anticipate the "generating a droplet" limitation of the '468 patent. As Dr. Dear explains, the '059 patent's discussion of droplet generation is separate from any discussion of mixing polynucleotides and barcode adaptors. CX-1827C at Q/A 427. Accordingly, Bio-Rad's anticipation argument fails for the same reasons discussed above for the "combining" limitation.

Bio-Rad's obviousness arguments for the "generating a droplet" limitation rely on the same combinations discussed above for the "combining" limitation. RIB at 180-83. Again, Bio-Rad fails to offer credible evidence for using the microfluidic systems disclosed in Colston, Samuels, Song, or Abate with the polynucleotides and barcode adaptors of the '059 patent. Accordingly, Bio-Rad's obviousness arguments fail to the same reasons discussed above for the "combining" limitation.

4. Secondary considerations of non-obviousness

For the same reasons discussed above in the context of the '024 patent, the success of 10X's domestic industry products further weigh against a finding of obviousness.

VI. THE '204 PATENT

A. Asserted Claims

10X is asserting claims 27, 29, 31, and 33 of the '204 patent. The asserted claims depend from unasserted independent claims 1, 23, and 25. Unasserted claim 1 recites:

> A composition comprising a plurality of capsules, said capsules situated within droplets in an emulsion, wherein said capsules are configured to release their contents into said droplets upon the application of a stimulus to provide said contents in said droplets in said emulsion,

> wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof.

Id., col. 44:42-48. Asserted claims 27 and 33 depend directly from claim 1. Claim 27 requires that the contents of each capsule "comprise at least 10,000 barcoded oligonucleotides releasably attached" to the capsule. *Id.*, col. 46:24-26. Claim 33 limits the claimed capsules to gel capsules. *Id.*, col. 46:42-43.

Unasserted claim 23 recites:

> A device comprising a plurality of partitions, wherein at least one partition of said plurality of partitions comprises a capsule, wherein said capsule is situated within a droplet in an emulsion, wherein said capsule is configured to release its contents into said droplet upon the application of a stimulus to provide said contents in said droplet in said emulsion, wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof.

Id., col. 45:51-58. Claim 29 depends directly from claim 23 and requires that the contents of the claimed capsule "comprise at least 10,000 barcoded oligonucleotides releasably attached" to the capsule. *Id.*, col. 46:30-32.

Unasserted claim 25 recites:

> A method comprising:
>
> a. providing a plurality of inner capsules, said inner capsules situated within outer capsules in an emulsion, wherein said inner capsules are configured to release their contents into said outer capsules upon the application of a stimulus, wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof; and
>
> b. providing a stimulus to cause said inner capsules to release their contents into said outer capsules in said emulsion.

Id., col. 46:3-12. Claim 27 requires that the contents of each capsule "comprise at least 10,000 barcoded oligonucleotides releasably attached" to the capsule. *Id.*, col. 46:36-38.

For the technical prong of the domestic industry requirement, in addition to the asserted claims, 10X relies on claim 10 of the '204 patent. Claim 10 depends from claim 1 through claims 2, 7, and 8. Claim 1 is recited above. Claim 2 requires that the capsules of claim 1 include "at least one of said capsules and said droplets comprise a species selected from the group consisting of a reagent and an analyte." *Id.*, col. 44:50-52. Claim 7 requires that the analyte of claim 2 be selected "from the group consisting of a cell, a polynucleotide, a chromosome, a protein, a peptide, a polysaccharide, a sugar, a lipid, a small molecule, and combinations thereof." *Id.*, col. 44:66-col. 45:2. Claim 8 requires the analyte of claim 7 to be a polynucleotide. *Id.*, col. 45:3-4. Claim 10 requires that the amount of polynucleotide in the composition of claim 8 be "sufficient to provide about 100-200X sequence coverage." *Id.*, col. 45:8-10.

B. Claim Construction

The parties agreed to construe "barcode" to mean a "label that may be attached to an analyte to convey identifying information about the analyte." Order No. 22 at 2. They agreed to construe "wherein said capsules are [capsule is] configured to release their [its] contents into said droplets [droplet] upon the application of a stimulus" to have its plain and ordinary meaning. *Id.*

C. Infringement

10X asserts that Bio-Rad's ddSEQ v1 [redacted] products infringe claims 27, 29, 31, and 33 of the '204 patent. With the exception of claim 33, the asserted claims of the '204 patent require a "capsule" or "capsules," wherein the contents of each capsule include barcode molecules that are "releasably attached" to the capsule. '204 patent, col. 44:42-49 (claim 1), col.

46:24-26 (claim 27), col. 46:30-32 (claim 29), col. 46:36-38 (claim 31). Bio-Rad's infringement argument relating to the "releasably attached" limitation are addressed above in the context of the '024 and '468 patents and are rejected for the same reasons in the context of the '204 patent. All of the asserted claims require a "capsule" or "capsules" that are "configured to release their contents into said droplets upon the application of a stimulus." *Id.*, col. 44:44-46 (claim 1), col. 46:5-7 (claim 25); *see also id.*, col. 45:53-56 (claim 23) ("wherein said capsule is configured to release its contents into said droplet upon the application of a stimulus to provide said contents in said droplet in said emulsion"). The parties agreed that the term "wherein said capsules are [capsule is] configured to release their [its] contents into said droplets [droplet] upon the application of a stimulus" did not need to be construed and should be given its "plain and ordinary meaning." Order No. 22 (Oct. 31, 2018) at 2. The claims further require that the stimulus be "selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof." *Id.*, col. 44:46-49 (claim 1), col. 45:56-58 (claim 23), col. 46:7-10 (claim 25).

For the reasons set forth below, the accused products do not literally infringe the asserted claims because they do not have a stimulus "selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof." In addition, 10X is estopped from relying on the doctrine of equivalents to show infringement.

1. Literal Infringement

The claims require that the capsules release their contents in response to a stimulus that is "selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof." '204 patent, col. 44:44-46 (claim 1), col. 45:44-58 (claim 23), col. 46:5-10 (claim 25). The recited stimuli form a "Markush group." In Markush

claims, alternative species or elements that can be selected as part of the claimed invention are listed as a group, called a Markush group. *Multilayer Stretch Cling Film Holdings, Inc. v Berry Plastics Corp.*, 831 F.3d 1350, 1357 (Fed. Cir. 2016) (citing *Abbott Labs. v. Baxter Pharm. Prods., Inc.,* 334 F.3d 1274, 1280 (Fed. Cir. 2003)). The term "group of" is traditionally used by patent drafters to signal a Markush group. *Id.* (citing *Gillette Co. v. Energizer Holdings, Inc.,* 405 F.3d 1367, 1372 (Fed. Cir. 2005)). Typically, Markush groups take the following form: "a member selected from the group consisting of A, B, and C." *Id.* (quoting *Gillette*, 405 F.3d at 1372) (internal quotation marks omitted). Each member of a Markush group is "alternatively usable for the puiposes of the invention." *Id.* at 1357-58 (quoting *In re Driscoll*, 562 F.2d 1245, 1249 (CCPA 1977)) (internal quotation marks omitted).

In the accused products, 10X argues that the barcode molecules are linked to the gel bead by "chemical bonds susceptible to [redacted] so that the barcode molecules are released when the [redacted]. CIB at 173. It is undisputed that the [redacted] are not one of the recited stimuli. *See, e.g.*, Tr. (Butte) at 371:24-372:17 (testifying that [redacted] by themselves are not a change in pH or ion concentration and do not reduce disulfide bonds). 10X, however, points to evidence showing that [redacted] *See, e.g.*, CX-0004C (Butte DWS) at Q/A 317-319. Relying on this evidence, 10X identifies the combination of the [redacted] as the claimed stimulus. CIB at 173. While Bio-Rad and Staff dispute 10X's contention that the presence of [redacted] the accused products still would not literally infringe the asserted claims.

While there is no dispute that the addition of [redacted] constitutes a "change in the ion concentration," which is a recited element of the Markush group, there is no evidence that the [redacted] would have any effect on the attached barcode molecules or the gel bead. *See, e.g.*, Tr. (Butte) at 474:18-21 ("Q: And you did not provide an opinion in your witness statement that [redacted] A: That's correct."). Rather, according to 10X's expert, Dr. Butte, [redacted] sever the barcode molecules from the gel bead. *See, e.g.*, CX-0004C (Butte DWS) at Q/A 318. Thus, as understood by Dr. Butte, the stimulus that causes the release of the barcode molecules from the gel bead in the accused products is the [redacted].

Dr. Butte's identification of the [redacted] in combination with a change in magnesium ion concentration for the claimed stimulus is legally flawed. By its express language—"wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof"—the Markush group at issue is limited to (1) one of the recited stimuli or (2) a combination of the recited stimuli; it does not encompass a combination of a recited stimulus and an unrecited

stimulus. In particular, the asserted claims define the Markush group as "consisting of" the recited stimuli and combinations thereof, as opposed to being "comprised" of the recited stimuli. "'Consisting of' is a term of patent convention meaning that the claimed invention contains only what is expressly set forth in the claim." *Multilayer,* 831 F.3d at 1358 (quoting *Norian Corp. v. Stryker Corp.,* 363 F.3d 1321, 1331 (Fed. Cir. 2004)) (internal quotation marks omitted). While the term "comprising" would have indicated that the group was open to additional, unrecited stimuli, the term "consisting of" indicates that unrecited stimuli are excluded from the group. *Id.* at 1358.

As explained by the Federal Circuit, "[t]he presumption that a claim term set off by the transitional phrase 'consisting of' is closed to unrecited elements is at least a century old and has been reaffirmed many times by our court and other courts." *Id.* While "the exceptionally strong presumption that a claim term set off with 'consisting of' is closed to unrecited elements" may be overcome if a patentee acts as his own lexicographer and "give[s] 'consisting of' an alternative, less restrictive meaning," the specification and prosecution history must "unmistakably manifest [such] an alternative meaning." *Id.* 10X does not contend that the patentees acted as their own lexicographers and re-defined "consisting of."

As shown in *Multilayer*, the closed nature of the claim language at issue excludes a combination of a recited stimulus (change in ion concentration) and an unrecited stimulus (enzymes). In *Multilayer,* the asserted claims were directed to a thermoplastic stretch wrap film having two outer layers and five inner layers. *Id.* at 1353. The claims further required that "five identifiable inner layers" be formed from materials selected from a Markush group "consisting of" various resins. *Id.* At issue was whether an inner layer composed of a combination of a recited resin and an unrecited resin fell outside the scope of the claimed Markush group. *Id.* at

1358. Answering the question in the affirmative, the Federal Circuit held that "construe[ing] the claims to cover any plastic film with five compositionally different inner layers, each of which contains any amount of one of the four recited resins," would "render the '055 patent's Markush language—each layer being selected from the group consisting of'—equivalent to the phrase 'each layer comprising one or more of.'" *Id.* at 1358.

10X has not pointed to any basis for distinguishing the closed Markush group at issue in *Multilayer* that would allow interpreting the Markush group at issue in this investigation to encompass a combination of a recited stimulus and an unrecited stimulus. 10X's only response to the argument that such a combination falls outside the scope of the claims, is to argue that the accused stimulus does not include the [redacted].

Relying on the [redacted] as the claimed stimulus has no legal basis and makes no logical sense. The claims require that the capsules release their contents in response to the claimed stimulus. *See* CIB at 181 ("The claimed function of applying a stimulus is to allow the release of the contents of a capsule into the droplet."). 10X does not argue and there is no evidence that [redacted] will cause the release of barcode molecules from the gel beads. [redacted].

10X's attempt to limit the accused stimulus to the [redacted]

Based on the foregoing, I find that the accused products do not literally infringe the asserted claims.

2. Doctrine of Equivalents

10X argues that the accused products satisfy the stimulus under the doctrine of equivalents ("DOE"). CIB at 181-84. In the accused products, the barcode molecules are released from the gel bead when the [redacted]. 10X, however, is precluded from relying on the DOE to satisfy the Markush group limitation.

Under the DOE, "a product or process that does not literally infringe upon the express terms of a patent claim may nonetheless be found to infringe if there is 'equivalence' between the elements of the accused product or process and the claimed elements of the patented invention." *Warner-Jenkinson Co. v. Hilton Davis Chem. Co.*, 520 U.S. 17, 21 (1997).

Although the DOE allows a "patentee to claim those insubstantial alterations that were not captured in drafting the original patent claim but which could be created through trivial changes," under prosecution history estoppel a patentee cannot use the DOE to recapture subject matter surrendered during prosecution. *Festo Corp. v. Shoketsu Kinzoku Kogyo Kabuskiki Co.*, 535 U.S. 722, 733-34 (2002) ("*Festo VIII*"). Such surrender occurs "[w]here the original application once embraced the purported equivalent but the patentee narrowed his claims to obtain the patent or to protect its validity." *Id.*

Making a narrowing amendment to secure a claim's issuance creates a presumption that prosecution history estoppel applies. *Id.* at 740-41. The presumption, however, is rebuttable as there may be some instances "where the amendment cannot reasonably be viewed as surrendering a particular equivalent." *Id.* Such situations include where the equivalent was unforeseeable at the time of the application or where the rationale underlying the amendment bears no more than a tangential relation to the equivalent in question. *Id.* at 741.

During the prosecution of the '204 patent, application claims 1, 78, and 110 matured into issued claims 1, 23, and 25, respectively. JX-0009.13630. In their original form, application claims 1 and 78 required a capsule (application claim 1) or capsules (application claim 78) "configured to release their contents . . . upon the application of a stimulus," but did not require that the stimulus be selected from a particular group of stimuli. *Id.* at .00080 (application claim 1); *see also id.* at .00085 (application claim 78) (requiring a capsule "configured to release its contents into said droplets upon the application of a stimulus"). Similarly, application claim 110 required a step of "providing a stimulus to cause said capsules to release their contents into said droplets," without requiring the stimulus be selected from a group of stimuli. *Id.* at .00087.

Although application claim 1 did not limit the stimulus to a group of stimuli, two of its dependent claims did. Application claims 19 and 21 depended directly from application claim 1. Application claim 19 required the stimulus to be "selected from the group consisting of a chemical stimulus, a bulk stimulus, a biological stimulus, a light stimulus, a thermal stimulus, a magnetic stimulus, and combinations thereof." *Id.* at .00081. Application claim 21 required the stimulus to be "selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof." *Id.* at .00081.

In an office action issued on January 29, 2016, the examiner rejected all of the pending claims as being anticipated in view of several prior art references. *Id.* at .09770-09781. Application claim 1 was found to be anticipated by seven references: (1) U.S. Patent Publication No. 2005/007951 to Berka *et al.* ("Berka"), (2) U.S. Patent Publication No. 2015/0079510 to Church *et al.* ("Church"), (3) U.S. Patent Publication No. 2014.0227706 to Kato et al. ("Kato"), (4) U.S. Patent Publication No. 2003/0207260 to Trnovsky et al. ("Trnovsky"), (5) U.S. Patent Publication No. 2013/0189700 to So *et al.* ("So"); (6) U.S. Patent Publication No. 2004/0258701 to Dominowski *et al.* ("Dominowski"); and (7) U.S. Patent Publication No. 2009/0025277 to Takanashi ("Takanashi") *Id.* at .09777-.099780. *Id.* at .09774-.099780. Application claim 19 was rejected as anticipated by five references: (1) Berka, (2) Trnovsky, (3) So, (4) Dominowski, and (5) Takanashi. *Id.* Application claims 78 and 110 were rejected as being anticipated by Berka. *Id.* Application claim 21 was rejected as being anticipated by Kato. *Id.*

On April 28, 2016, the applicants responded to the rejections by, *inter ilia*, cancelling application claims 19 and 21 and amending application claims 1, 78, and 110. As amended, application claims 1, 78, and 110 incorporated application claim 21's limitation requiring that the stimulus be "selected from the group consisting of a change in pH, a change in ion concentration,

reduction of disulfide bonds, and combinations thereof." *Id.* at .10009 ("[C]laims 1, 31, 78, 89, 110 and 118 have been amended to recite 'wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof,' thus incorporating the elements of Claim 21."); *see also id.* at .10000, .10002, .10003. With this amendment, the applicants argued that the amended application claims were allowable over the cited prior art with the exception of Kato. *Id.* at .10009 ("Initially, as Claim 21 was rejected only over Kato, Applicant understands that the Office acknowledges that none of Berka, Church, Trnovsky, So, Dominowski and Takanashi teach or disclose 'wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof,' as recited in claims 1, 31, 78, 89, 110 and 118."). With regard to Kato, the applicants argued that "Kato does not teach or disclose, 'wherein said capsules are configured to release their contents **into said droplets** upon the application of a stimulus,' as recited in Claim 1." *Id.* at .10010. The applicants also argued that Kato did not qualify as prior art. *Id.*

On August 5, 2016, the examiner rejected the amended claims in view of a new set of prior art references and noted that the previous rejections had been rendered moot in view of the new grounds of rejection. *Id.* at .10074. The examiner also "noted that the 102(b) rejection of Claims 1 and 21 over Kato has been withdrawn in light of the applicant's persuasive arguments." *Id.* In response to the new rejections, the applicants further amended application claims 1, 78, and 110 to require that the capsule or capsules "provide said contents in said droplets in said emulsion" upon the application of a stimulus. *Id.* at .10118, .10120-.10121. The application claims as amended were allowed. *Id.* at .13617.

10X argues that "[i]nfringement under the doctrine of equivalents is not barred here by the prosecution history estoppel because there was no disclaimer during prosecution of all chemical, bulk, or biological stimuli." CIB at 182. 10X's argument, however, is unpersuasive. A narrowing amendment made in order to gain issuance triggers the presumption that a patentee is estopped from relying on the DOE to show infringement. *Festo VIII,* 535 U.S. at 740-41. It is indisputable that the April 28, 2016 amendments to application claims 1, 78, and 110 narrowed the scope of the claims. As originally drafted, the application claims did not require the claimed stimulus to be selected from any group of stimuli, much less from the "group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof." It is also indisputable that the narrowing amendment was made to overcome prior art as the applicants expressly cited the amendment to overcome several prior art references relied upon by the examiner: "[A]s Claim 21 was rejected only over Kato, Applicant understands that the Office acknowledges that none of Berka, Church, Trnovsky, So, Dominowski and Takanashi teach or disclose 'wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof,' as recited in claims 1, 31, 78, 89, 110 and 118." JX-0009 at .10009

Thus, the applicants' narrowing amendment triggers the presumption that 10X is estopped from relying on the DOE to show that accused products satisfy the stimulus limitation. *See Festo VIII,* 535 U.S. at 740-41. 10X attempts to rebut the presumption by arguing that the narrowing amendment was tangential to the alleged equivalent, which 10X identifies as [redacted]

[redacted]

different Markush element, [redacted] The only basis that 10X has put forth in support of its contention that the amendment was tangential to the alleged equivalent is that the alleged equivalent was not disclosed in the prior art references relied upon by the examiner to reject the pending claims. This argument, however, fails on examination.

"[A]n amendment made to avoid prior art that contains the equivalent in question is not tangential; it is central to allowance of the claim." *Festo Corp. v. Shoketsu Kinzoku Kogyo Kabuskiki Co., Ltd.*, 344 F.3d 1359, 1369-70 (Fed. Cir. 2003) ("*Festo IX*").[14] It is 10X's burden to show that the reason for the amendment is tangential to the alleged equivalent. *Id.* at 1369. Moreover, the reason for the amendment must be "objectively apparent" from the prosecution history "without the introduction of additional evidence, except, when necessary, testimony from those skilled in the art as to the interpretation of that record." *Id.; see also Integrated Tech. Corp. v. Rudolph Techs., Inc.*, 734 F.3d 1352, 1358. This is a burden that 10X has not met.

At the hearing, 10X's own expert Dr. Butte confirmed that one of the references in question, Trnovsky, discloses the use of the enzyme agarase as a stimulus. Tr. 431:14-16. Describing Trnovsky as a "complicated paper," Dr. Butte further testified that he was unable to "tell one way or the other whether" Trnovsky disclosed the use of ion cofactors with agarase. *Id.* at 431:17-25. Dr. Butte, however, acknowledged that Trnovsky discloses "buffers used with

[13] In its initial post-hearing brief, 10X also argued in the alternative that [redacted]. In its reply brief, however, 10X appears to have abandoned this argument. *See* CRB at 84-85.

[14] The converse is not necessarily true. *Integrated Tech. Corp. v. Rudolph Techs. Inc.*, 734 F.3d 1352, 1358 (Fed. Cir. 2013) ("It does not follow . . . that equivalents not within the prior art must be tangential to the amendment.") (quoting *Chimie v. PPG Indus. Inc.*, 402 F.3d 1371, 1383 (Fed. Cir. 2005)) (internal quotation marks omitted).

agarase which may provide a cofactor and that there may be an ion cofactor." *Id.* at 432:1-5. This testimony by its own expert is fatal to 10X's argument that the reason for the narrowing amendment is tangential to the alleged equivalent.

10X's only response to its expert's testimony is to characterize it as "hypothetical testimony" and argue that surrender of the alleged equivalent must be shown through "the actual prior art disclosure and amendment in prosecution." CRB at 85 n. 36.[15] It is 10X's burden, however, to show that the narrowing amendment is tangential to the alleged equivalent, not Staff's and Bio-Rad's burden to show the converse. *Festo IX*, 344 F.3d at 1369-70. 10X would be unable to meet its burden, even if its expert's testimony on the issue was discounted in its entirety as "hypothetical testimony." This is because the record is devoid of any evidence concerning Trnovsky's teachings. As 10X acknowledges, Trnovsky and the other references relied upon by the examiner are not in evidence. CRB at 85. Nor does the prosecution history describe Trnovsky's disclosure in sufficient detail to determine whether the narrowing amendments are tangential to the alleged equivalent. In rejecting application claims 1 and 19 in view of Trnovsky, the examiner did not describe the reference's teachings, but cited particular portions of Trnovsky. For example, with respect to application claim 19, the examiner's rejection reads as follows:

> **Claim 19** is drawn, in part, to an embodiment of the composition of Claim 1 wherein said stimulus is selected from a defined group consisting of a chemical stimulus, a bulk stimulus and a biological stimulus.

[15] The Federal Circuit has held that it is appropriate to rely on "testimony from those skilled in the art as to the interpretation of" the prosecution history "when necessary." *Festo IX,* 344 F.3d at 1369-70. Such testimony is appropriate for a "complicated paper," such as Trnovsky. Tr. (Butte) at 431:17-25.

Trnovsky *et al.* teach these limitations, see ¶s 9 and 102.

JX-0009 at .09778; *see also id.* at .09777 ("Trnovsky et al. teach a composition comprising all of the limitations of **Claims 1-2, 4-5, 7-10, 13 and 28-30** see at least the abstract, as well as, ¶s 32, 84, 88, 99 and 102). Nor does the applicants' response provide any information about Trnovsky's disclosure other than Trnovsky does not disclose the recited stimuli. JX-0009 at .10009 ("[A]s Claim 21 was rejected only over Kato, Applicant understands that the Office acknowledges that none of Berka, Church, Trnovsky, So, Dominowski and Takanashi teach or disclose 'wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof,' as recited in claims 1, 31, 78, 89, 110 and 118.").

Based on the foregoing, I find that 10X has not shown that the reason for the narrowing amendment is tangential to the alleged equivalent. Accordingly, 10X is estopped from relying on the DOE to show that the stimulus limitation is satisfied by the accused products.

D. Domestic Industry

10X asserts that each of its domestic industry products practice claims 10, 27, 29, 31, and 33 of the '204 patent. CIB at 187-188. 10X's contentions regarding the practice of the '204 patent by its domestic industry products are undisputed by both Bio-Rad and Staff. SIB at 86 (arguing that the DI products practice the claims at issue); RIB at 136-64 (not addressing the technical prong with respect to the '204 patent). As set forth below, I find that 10X's linked-read DI products practice claims 10, 27, 29, 31, and 33 of the '204 patent and 10X's single cell DI products practice claims 27, 29, 31, and 33 of the '204 patent.

1. Claim 10

Claim 10 depends from claim 1 through claims 2, 7, and 8. Claim 1 consists of a preamble and three limitations. To the extent that the preamble is limiting, 10X's DI products provide a "composition." CX-0004C (Butte DWS) at Q/A 369-370. As required by first limitation of claim 1, the DI products provide a plurality of capsules in the form of gel beads. *Id.* at Q/A 372. In accordance with the second limitation of claim 1, the gel beads are situated within droplets in an emulsion. *Id.* at Q/A 374; CX-0538.00002 ("A GEM is a '**G**el bead in **EM**ulsion' droplet that encapsulates each tiny micro-reaction within the Chromium System. Here we show a Single Cell GEM with a single T-cell, reagents and barcoded gel bead all partitioned within a single oil droplet."). As required by third limitation, the gel beads are configured to release their contents (barcoded primers) into the droplets upon application of [redacted], which [redacted] connecting the barcoded primers to the gel beads. CX-0004C (Butte DWS) at Q/A 376, 378.

As required by claims 2, 7, and 8, in 10X's single-cell DI products, droplets encapsulating a cell containing a plurality of mRNA (a claimed analyte and a polynucleotide) are formed. *Id.* at Q/A 381, 384, and 387. In 10X's linked-read DI products, droplets containing gDNA molecules (a claimed analyte and a polynucleotide) are formed. *Id.*

As required by claim 10, the amount of gDNA provided by 10X's linked-read DI products is sufficient to provide about 100-200X sequence coverage. *Id.* at Q/A 390. 10X, however, does not address how the single-cell DI products satisfy the limitation of claim 10.

Based on the foregoing, I find that 10X's linked-read DI products practice claim 10, but that 10X has failed to show that its single-cell DI products practice claim 10.

2. Claims 27 and 33.

Claims 27 and 33 depend from claim 1, which is discussed above with respect to claim 10. As required by claim 27, each gel bead in 10X's domestic industry products contains millions of barcoded primers that are releasably attached to the bead. *Id.* at Q/A 392. As required by claim 33, the capsules (gel beads) in the domestic industry products are made of a gel. *Id.* at Q/A 370, 372, 374, 376, and 378.

Based on the foregoing, I find that 10X's linked-read DI products and single cell DI products practice claims 27 and 33.

3. Claim 29

Claim 29 depends from claim 23. Claim 23 is consists of a preamble and three limitations. To the extent the preamble is limiting, 10X's DI products provide a "composition." CX-0004C (Butte DWS) at Q/A 395. As required by first limitation of claim 1, the DI products provide a plurality of partitions in the form of gel beads. *Id.* at Q/A 372, 397. As further required by the first limitation, each gel bead is a capsule. *Id.* In accordance with the second limitation, each gel bead is situated within a droplet in an emulsion. *Id.* at Q/A 374, 399; CX-0538.00002 ("A GEM is a '**G**el bead in **EM**ulsion' droplet that encapsulates each tiny micro-reaction within the Chromium System. Here we show a Single Cell GEM with a single T-cell, reagents and barcoded gel bead all partitioned within a single oil droplet."). As required by the third limitation, the gel beads are configured to release their contents in the form of barcoded primers into the droplets upon application of [redacted], which [redacted] connecting the barcoded primers to the gel beads. CX-0004C (Butte DWS) at Q/A 376, 378, 400. As required by claim 29, each gel bead in 10X's domestic industry products contains millions of barcoded primers that are releasably attached to the bead. *Id.* at Q/A 392, 402.

Based on the foregoing, I find that 10X's linked-read DI products and single cell DI products practice claim 29.

4. Claim 31

Claim 31 depends from claim 25. Claim 25 is a method claim consisting of a preamble and two steps. To the extent the preamble is limiting, 10X's DI products perform a method. CX-0004C (Butte DWS) at Q/A 405. As required by first step of claim 25, the DI products provide a plurality of capsules in the form of gel beads. *Id.* at Q/A 372, 407. Each gel bead is situated within a droplet in an emulsion. *Id.* at Q/A 374, 407; CX-0538.00002 ("A GEM is a '**Ge**l bead in **EM**ulsion' droplet that encapsulates each tiny micro-reaction within the Chromium System. Here we show a Single Cell GEM with a single T-cell, reagents and barcoded gel bead all partitioned within a single oil droplet."). The gel beads are configured to release their contents (barcoded primers) into the droplets upon application of [redacted], which [redacted] connecting the barcoded primers to the gel beads. CX-0004C (Butte DWS) at Q/A 376, 378, 407. As required by the second step, 10X's domestic industry products apply a stimulus to the gel beads provided in the first step, resulting in the gel beads releasing their contents, the barcode primers. *Id.* at Q/A 376, 378, 409. As required by claim 31, each gel bead contains millions of barcoded primers that are releasably attached to the bead. *Id.* at Q/A 392, 411.

Based on the foregoing, I find that 10X's linked-read DI products and single cell DI products practice claim 31.

E. Invalidity

Bio-Rad contends that the asserted claims of the '204 patent are invalid as anticipated or rendered obvious by the '059 patent and/or the Church patent, alone or in combination with

additional prior art. RIB at 145-56. The asserted claims of the '204 patent require either barcode molecules that are releasably attached to a capsule (claims 27, 29, and 31) or a gel bead that is "configured to release" its contents (claim 33). Accordingly, the parties' arguments regarding invalidity for the '204 patent are substantially identical to those addressed above in the context of the "releasable attachment" limitation of the '024 patent. *See* SRB at 38-39. For the same reasons discussed above, Bio-Rad has failed to show that any asserted claim of the '204 patent is anticipated and/or rendered obvious by the '059 patent and/or the Church patent because these references do not disclose the "releasably attached" or "configured to release" limitations. Moreover, the success of 10X's domestic industry products further weigh against a finding of obviousness.

VII. THE '530 PATENT

The '530 patent issued on January 2, 2018, naming inventors Benjamin Hindson, Serge Saxonov, Kevin Ness, Paul Hardenbol, Mirna Jarosz, and Michael Schnall-Levin. JX-0007.

A. Asserted Claims

10X is asserting claims 1, 4, 11, 14, 19, 26, and 28 of the '530 patent. Claim 1 is independent and the remaining claims depend directly or indirectly from claim 1. Claim 1 recites:

> A method for nucleic acid preparation or analysis, comprising:
>
> (a) providing:
>
> (i) at least 1,000 gel beads;
>
> (ii) releasably attached to each of said at least 1,000 gel beads, at least 1,000 barcode molecules comprising identical barcode sequences that are distinct from barcode sequences of at least 1,000 barcode molecules releasably attached to any other gel bead of said at least 1,000 gel beads; and

(iii) a plurality of cells each comprising a plurality of polynucleotide molecules;

(b) generating a plurality of droplets, wherein at least 1,000 droplets of said plurality of droplets each comprise:

(i) a single gel bead from said at least 1,000 gel beads; and

(ii) a single cell from said plurality of cells; and

(c) in each of said at least 1,000 droplets, using said plurality of polynucleotide molecules from said single cell and barcode molecules of said at least 1,000 barcode molecules from said single gel bead to generate a plurality of barcoded polynucleotide molecules,

wherein said barcode molecules become detached from said gel bead.

Id., col. 47:58-67, col. 48:57-col. 49:4.

Claim 4 depends from claim 1 through unasserted claim 3. Claim 3 requires that the polynucleotide molecules be mRNA. *Id.*, col. 49:8-10. Claim 4 further requires that the barcoded polynucleotide molecules be generated by reverse transcribing the mRNA in the presence of the barcode molecules. *Id.*, col. 49:11-14. Claim 19 depends from claim 1 through unasserted claim 17. Claim 17 requires that the barcode molecules "comprise combinatorial assemblies of sequences from sequence modules." *Id.*, col. 50:5-7. Claim 19 further requires that each of the combinatorial assemblies comprise a first sequence, a second sequence, and a third sequence. *Id.*, col. 50:13-15.

Claims 11, 14, 26, and 28 depend directly from claim 1. Claim 11 requires that the barcode molecules in each of the droplets be released from a single gel bead. *Id.*, col. 49:34-36. Claim 14 requires that each gel bead have "disposed within" it at least 1,000 barcode molecules. *Id.*, col. 49:44-45. Claim 26 requires that each gel bead contain at least 1,000,000 barcode

molecules. *Id.*, col. 50:30-31. Claim 28 requires that the barcode molecules become detached before the generation of the barcoded polynucleotide molecules. *Id.*, col. 50:35-37.

B. Claim Construction

The parties agreed to construe "barcode" to mean a "label that may be attached to an analyte to convey identifying information about the analyte." Order No. 22 at 2. They agreed that the term "wherein said barcode molecules become detached from said gel bead" has its lpain and ordinary meaning. *Id.* In the *Markman* order, the term "amplifying" was construed to mean "increasing the number of copies of the target sequence to be detected," including by reverse transcription. *Id.* at 31-45. The terms "providing," "said at least 1,000 droplets," and "a plurality of cells" were given their plain and ordinary meaning, with a requirement that all of the "at least 1,000 droplets" in the second step be generated before the third step of the claim is performed on any of "said at least 1,000 droplets." *Id.* at 45-51. In Order No. 35, this claim construction was further clarified so that it does preclude the generation of some barcoded molecules before the start of the claimed third step. Order No. 35 at 4-6 (Mar. 5, 2019).

C. Infringement

10X is asserting claims 1, 4, 11, 14, 19, 26, and 28 of the '530 patent against Bio-Rad's "ddSEQ Cartridges (v1 [redacted]), ddSEQ Single-Cell Isolator (v1 [redacted]), ddSEQ Cartridge Holder, and consumables and assays used with and/or as part of Bio-Rad's ddSEQ v1 [redacted]2 products including SureCell WTA 3′ (also referred to as WTA 3′ v1), [redacted]

[redacted]

1. Claim 1

As discussed in the *Markman* order, claim 1 is directed to a three-step method. Order No. 22 (Oct. 31, 2018) at 44. The first step requires "providing" at least 1,000 gel beads with

"releasably attached" barcode molecules and "a plurality of cells" containing polynucleotides. '530 patent, col. 47:60-67 & col. 48:58-64. The second step requires generating "a plurality of droplets, wherein at least 1,000 droplets of said plurality of droplets each" have "a single gel bead from said plurality of cells" and "a single cell from said plurality of cells." *Id.*, col. 48:60-64. The third step requires using the polynucleotide molecules and barcode molecules to form "a plurality of barcoded polynucleotide molecules" "in each of said 1,000 droplets." *Id.*, col. 48:65-col. 49:4. As found in the *Markman* Order, the second step of generating "at least 1,000 droplets" must be completed before the third step of generating a "plurality of barcoded polynucleotide molecules" is performed in any of the droplets. Order No. 22 (Oct. 22, 2018) at 51.

a. Preamble

To the extent that the preamble is limiting, there is no dispute that the accused products are methods of nucleic acid preparation and analysis or are used in such methods. '530 patent, col. 47:58-59. Specifically, Bio-Rad's ddSEQ v1 products are used with the WTA 3'v1 assay to prepare the mRNA of a single cell for single cell whole transcriptome analysis. *See* CX-0004C (Butte DWS) at Q/A 68 (describing the release and barcoding of mRNA from cell in the WTA 3′ v1 assay's workflow). [redacted] *See* CX-0004C (Butte DWS) at Q/A 68 (describing the release and barcoding of mRNA from cell in the WTA 3′ v1 assay's workflow), 84 [redacted]

performs the same work flow as the WTA 3′ v1 assay to partition and barcode the mRNA transcripts of individual cells."), 93 (In the scATAC-seq assay, "[t]he oligonucleotide barcodes are released from the gel bead [redacted] and attach to the genomic DNA fragments for amplification through PCR in the droplet"), 100 ([redacted]; JX-0091C.00006-.00007 (describing the workflow of the scATAC-seq assay); CX-1491C.00013-.00016 (describing the WTA 3′ v1 assay); JX-0105C.00024 ([redacted])

b. Step 1: "providing" a plurality of cells and at least 1,000 gel beads

The first step of claim 1 requires "providing" at least 1,000 gel beads and a plurality of cells. '530 patent, col. 47:60-67 & col. 48:57-58. Each gel bead must have "releasably attached" to it "at least 1,000 barcode molecules comprising identical barcode sequences." *Id.* at col. 47:62-67. The barcode sequences of barcode molecules attached to each bead must be distinct from the barcode sequences of the barcode molecules attached to any other bead. *Id.* Each cell must "compris[e] a plurality of polynucleotide molecules." *Id.* at col. 48:57-58.

There is no dispute that the accused products can be used to provide at least 1,000 gel beads and a plurality of cells. The accused products use gel beads. CX-0004C at Q/A 489 ("[T]he ddSEQ v1 products provide gel beads composed of polyacrylamide and users provide these gel beads in performing the claimed method."), 491 ([redacted]). The accused products have the ability to provide at least 1,000 gel beads

and a corresponding number of cells. CX-0004C (Butte DWS) at Q/A 490 (testifying that Bio-Rad's ddSEQ v1 products provide at least 1,000 beads and an equal number of cells); 492 [redacted], 493 (testifying that Bio-Rad's scATAC-seq assay using the v1 cartridge provide at least 1,000 beads and an equal number of cells); JX-0036.00002-00003 (data sheet showing that 1,384 single cells were barcoded in one WTA 3′ v1 assay); CX-1573C (18,000 cells processed in WTA 3′ v1 assays); CX-1529C.00037 [redacted].

The cells provided by the accused products contain a plurality of polynucleotide molecules. CX-0004C (Butte DWS) at 502 (testifying that the cells provided by the ddSEQ v1 products comprise a plurality of mRNA molecules), 504 ([redacted]

Although Bio-Rad disputes whether the barcode molecules are releasably attached to the gel beads, here is no dispute that each of the gel beads has at least 1,000 barcode molecules attached to it. CX-0004C (Butte DWS) at 496; JX-0036.00004 (showing at least 3,000 genes are detected per cell and thus confirming at least 3,000 barcode molecules per bead). The barcode molecules have barcode sequences in the form of oligonucleotide molecules. CX-0004C (Butte DWS) at Q/A 127 (testifying that "oligonucleotide molecules released from the gel beads in Bio-Rad's ddSEQ v1 products each include a Cell Barcode sequence") ('024 patent, claim 1),

130 ([redacted]), 131 (testifying that "[t]he claimed oligonucleotide molecules in the ATAC-seq assay" include a barcode sequence). The barcode molecules attached to each gel bead have barcode sequences that are distinct from the sequences of barcode molecules attached to other gel beads. CX-0004C (Butte DWS) at Q/A 497. In particular, for the ddSEQ v1 products, there are almost [redacted] pools of barcode molecules and each pool of barcode molecules has a unique barcode sequence. *Id.*; JX-0050C.00026; CX-0018C (Lebofsky Depo. Tr.) at 115:13-116:4. [redacted]. CX-0004C (Butte DWS) at Q/A 497; CX-0009C (Agresti Dep. Tr.) at 437:1-7.

The barcode molecules are releasably attached to the gel bead through a [redacted] CX-0004C (Butte DWS) at Q/A 143 (ddSEQ v1 products), 160 ([redacted]); JX-0036.00001 ("Comprehensive Single-Cell RNA Sequencing Workflow"). Bio-Rad disputes that its products satisfy the "releasably attached" requirement for the same reasons that it contested that the requirement was satisfied with respect to the asserted claims of the '024 and '468 patents. Bio-Rad's argument is rejected for the same reasons that it was rejected with respect to the '024 and '468 patents.

For the foregoing reasons, I find that the accused products satisfy the first step of claim 1.

c. Step 2: "generating a plurality of droplets"

The second step of claim 1 requires generating a plurality of droplets, wherein at least 1,000 of the droplets comprise a "single gel bead" and a "single cell." '530 patent, col. 48 59-64. The accused products are capable of producing a plurality of droplets. CX-0004C (Butte DWS)

at Q/A 508-509; CX-1357C.00018; JX-0050C.00014 (ddSEQ v1 products provide between 50,000-75,000 droplets for each sample, and about 260,000 droplets per chip); CX-1529C.00037; JX-0110C.00006 ([redacted]); CX-0018C at 197:24-198:5.

The accused systems can be used to generate at least 1,000 droplets with a cell and gel bead. When all four lanes are primed with a Cell Suspension Mixture, the ddSEQ v1 cartridge can generate approximately 1,200 droplets having a cell and a gel bead. CX-0004C (Butte DWS) at Q/A 512; JX-0035.00011 (requirement of 40,000 input cells for 1,200 processed cells); CX-0016C (Kaihara Depo. Tr.) at 166:21-167:17 (testifying that most users have [redacted] to input into a ddSEQ v1 cartridge); JX-0036.00002–03 (1,384 droplets containing a single cell and a gel bead generated using one ddSEQ v1cartridge); CX-1494C.00016 ([redacted]). If the scATAC-seq assay is performed using the ddSEQ v1 cartridge, each lane is capable of generating 500 droplets with a cell and gel bead. CX-0016C (Kaihara Depo. Tr.) at 155:15-158:25; CX-0004C (Butte DWS) at Q/A 515-516. [redacted]. CX-0004C (Butte DWS) at Q/A 513; CX-1529C.00037.

Bio-Rad does not dispute that the accused products are capable of generating at least 1,000 droplets containing a cell and gel bead. Instead, Bio-Rad argues that the accused products do not satisfy the second step of claim 1 because they do not generate a "collection" of at least 1,000 of such droplets. RIB at 194-95. Bio-Rad argues that droplets are formed one-by-one in each chamber of the ddSEQ v1 [redacted] cartridge and, after each droplet is formed, the cell in the droplet is "destroyed almost immediately." RX-0665C (Metzker RWS) at Q/A 100 ("Think of it

this way, as droplet number one forms with a cell and a gel bead in it, the cell is destroyed almost immediately because the cell lysis reagent acts on the cell membrane. So, you never form a collection of 1,000 droplets containing a single cell and a single gel bead before any barcoding begins."). As a result, Bio-Rad argues, that there is never an instant of time where there is at least 1,000 droplets with a cell and gel bead. Bio-Rad's non-infringement argument reads a limitation into the claim that is not present, *viz.*, that a collection of at least 1,000 droplets with a cell and gel bead must exist in some instant of time.

Neither the claim language nor the *Markman* order require amassing such a "collection." The claim language and *Markman* order only require that all of the droplets be generated prior to proceeding to the third step. '530 patent, col. 48:59-64 ("generating a plurality of droplets, wherein at least 1,000 droplets of said plurality of droplets each comprise" a single gel bead and a single cell); Order No. 22 (Oct. 31, 2019) at 48 ("The second step of claim 1's three-step method requires the generation of 'at least 1,000 droplets' . . . ."). Although the step of generating droplets with a cell and gel bead must be completed before the start of the third step, the third step does not require at least 1,000 droplets having a cell and a gel bead. The third step requires at least 1,000 droplets containing (1) a plurality of polynucleotide molecules from a single cell and (2) the barcode molecules from a single bead. '530 patent, col. 48:65-col. 49:2. Therefore, even if the cells are lysed almost immediately after droplet formation so that there is never more than a handful of droplets with a cell and gel bead at any single point in time, the claim language is still satisfied so long as at least 1,000 of such droplets had been generated before the start of the third step.

Based on the foregoing, I find that the accused products satisfy the second step of claim 1.

d. Step 3: generating a plurality of "barcoded polynucleotide molecules"

The third step requires that, in each of said at least 1,000 droplets, a plurality of barcoded polynucleotide molecules be generated using the polynucleotide molecules from the cell and the barcode molecules from the gel bead. '530 patent, col. 48:65-col. 49:2. The step further requires that the "barcode molecules become detached from said gel bead." *Id.* at col. 49:3-4.

There does not appear to be a dispute that in each droplet containing a single cell and single gel bead the following processes occur: (1) the cell lyses and releases polynucleotide molecules in the form of mRNA or gDNA into the droplet; (2) [redacted] the barcode molecules from the gel bead; (3) the released barcode molecules bind with either mRNA (WTA 3′ v1, [redacted]) or tagmented gDNA fragments (scATAC-seq assay); and (4) the barcode molecules and polynucleotide molecules are used as templates to generate either barcoded cDNA (WTA 3′ v1, [redacted]) or barcoded gDNA (scATAC-seq assay).

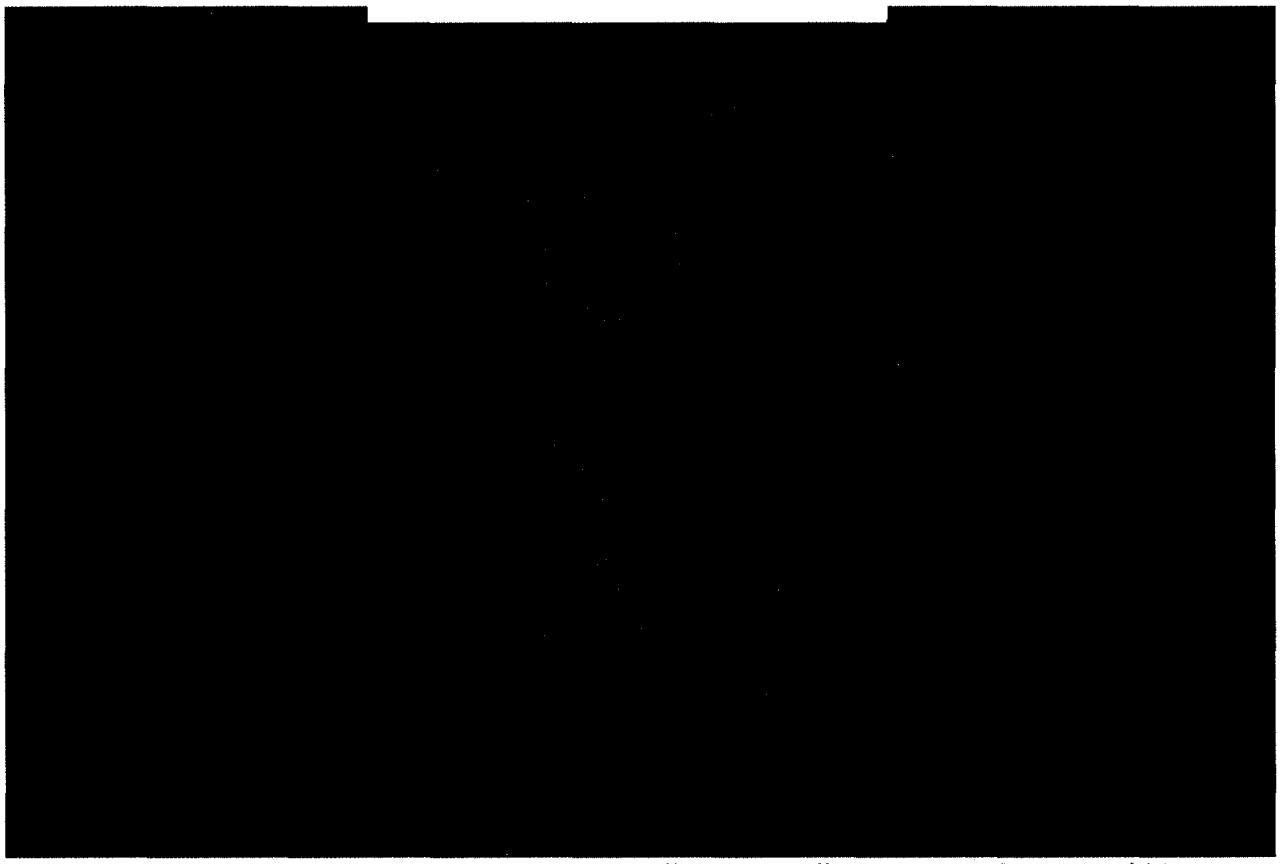
[redacted]

JX-0075C.00018 (describing the WTA 3′ v1 assay); *see also* JX-0074C.00009 (describing the WTA 3′ v1 assay); JX-0088C.00015 (describing the WTA 3′ v1 assay); JX-0091C.00020

(describing the scATAC-seq assay); CX-1491C.00018 (describing the scATAC-seq assay); JX-0034.00027, 00031, 00037 CX-0004C (Butte DWS) at Q/A 198-200, 204-209, 519, 523; CX-0018C (Lebofsky Depo. Tr.) at 154:7-157:22, 160:8-161:18; CX-0019C (Norton Depo. Tr.) at 194:24-195:13.

According to 10X, the third step occurs when the droplets are heated on a thermal cycler. Bio-Rad argues that 10X has not shown that a plurality of polynucleotide molecules are barcoded in each of at least 1,000 droplets while the droplets are being incubated on the thermal cycler. RIB at 196. According to Bio-Rad, the enzymes in the droplets "are active and start reacting to form barcoded molecules immediately upon droplet formation" and suggests—but does not state—that all of the barcoding is completed in a subset of the droplets prior to incubation, so that barcoded polynucleotides are generated in less than 1,000 droplets during the incubation step. *Id.*

With regard to the WTA 3′ v1, [redacted], Bio-Rad's documentation indicates that barcoded cDNA is generated when the droplets are incubated in accordance to the thermal cycler's "Reverse Transcription (RT) program."



JX-0088C.00015; *see also* JX-0034.00043 (SureCell WTA 3' Library Prep Reference Guide) ("Cell lysis and cell barcoding of mRNA transcripts takes place in each droplet during reverse transcription."), 00025 ("**Reverse Transcribe Samples** This step reverse transcribes samples on a thermal cycler,"), .00026 ("Save the following Reverse Transcription (RT) program on a thermal cycler . . . .").

The thermal cycler's reverse transcription program heats the droplets at 37°C for 30 minutes and then heats the droplets at 50°C for 60 minutes. JX0034.00026. [redacted]. This evidence supports 10X's position that the following processes occur in the thermal cycler: (1) the release of the barcode molecules from the gel bead and (2) the generation of barcoded polynucleotides through reverse transcription.

With regard to the scATAC-seq assay, Dr. Ronald Lebofsky—who holds the position R& D Manager II at Bio-Rad and [redacted]—confirmed that barcoded polynucleotides are generated during the first heating cycle of the thermal cycler. CX-0018C (Lebofsky Depo. Tr.) at 157:8-16, 159:24-160:9.

Bio-Rad dismisses the statements in its own documents as "general statements," but does not point to any persuasive evidence countering those statements. RIB at 109. Bio-Rad primarily relies on the testimony of its expert Dr. Metzger, who testifies that [redacted] RX-0665C (Metzker RWS) at Q/A 97-98. Dr. Metzer also testifies that [redacted] the barcode molecules from the gel bead soon after the droplet is formed. *Id.* Dr. Metzker, however, does not state that these processes are completed before the droplets are incubated on the thermal cycler, only that the processes start before incubation. *Id.* at Q/A 97-108. In support of its argument, Bio-Rad also points to the hearing testimony of 10X's expert, who testified that the reverse transcriptase used in the accused products may exhibit a "small element" of activity at room temperature. Tr. (Butte) at 397:7-12. As discussed above, however, Bio-Rad's own documents clearly show that [redacted] primarily occur during incubation.

Assuming *arguendo* that Dr. Metzker is correct and [redacted] and reverse transcriptase are active as soon as droplets are formed in the single-cell isolator, the enzymes would be active only for a relatively short period of time at a suboptimal temperature. The single-cell isolator operates at room temperature (~20°C) and completes a run within five

minutes. CX-0004C (Butte DWS) at Q/A 526; JX-0075C.00016; JX-0034 ("Single-cell isolation begins automatically after the ddSEQ Single-Cell Isolator door is closed and takes approximately 5 minutes."). In that period of time, for Bio-Rad's argument to hold true, the [redacted] and reverse transcriptase must not only be active, but they must reach a point where all the barcoded molecules have been cleaved from the gel bead and/or the reverse transcriptase has finished forming barcoded cDNA in a sufficient number of droplets so that these processes occur in less than 1,000 droplets during incubation. As shown by their product labels, however, room temperature is a suboptimal temperature for both the [redacted] and the reverse transcriptase used in the accused products. The reaction temperature of the [redacted] (37°C) is significantly higher than room temperature (20°C) and the optimal reaction temperature of the reverse transcriptase is higher still (50-55°C). JX-0050C.00056. Moreover, the period of time that droplets are being generated in the single-cell isolator is short relative to the periods of time that the droplets are being incubated. Specifically, the droplets are incubated at 37°C (the [redacted] reaction temperature) for 30 minutes and then heated at 50°C (the reverse transcriptase's optimal reaction temperature) for another 60 minutes. JX-0034C.00026. There is no evidence suggesting that the single-cell isolator'sgive-minute run-time provides the enzymes sufficient time to finish catalyzing their reactions within the droplets, especially at a suboptimal temperature.

On the basis of this evidence, I find that 10X has shown by the preponderance of the evidence that at least the bulk of the following processes occur while the droplets are being heated on the thermal cycler: (1) the [redacted] release the barcode molecules from the gel bead and (2) the reverse transcription of barcoded cDNA from mRNA and barcode molecules. Accordingly, I find that the accused products satisfy the third step of claim 1 and infringe

claim 1.

2. Claims 4, 11, 14, 19, 26, and 28

Claim 4, 11, 14, 19, 26, and 28 depend either directly or indirectly from claim 1.

a. Claim 4

Claim 4 depends from claim 1 through unasserted claim 3. Claim 3 requires that the polynucleotide molecules be mRNA. '530 patent, col. 49:8-10. Claim 4 further requires that the third step of claim 1 comprise reverse transcribing "said plurality of mRNA molecules in presence of said barcode molecules to generate said plurality of barcoded polynucleotide molecules." '530 patent, col. 49:11-14. As discussed above, the WTA 3′ v1, WTA 3′ v2, and CITE-seq assays generate barcoded cDNA by reverse transcribing mRNA in the presence of barcode molecules. *See, e.g.,* JX-0075C.00018 (describing the WTA 3′ v1 assay); JX-0074C.00009 (describing the WTA 3′ v1 assay); JX-0088C.00015; JX-0034.00027, 00031, 00037; CX-0004C (Butte DWS) at Q/A 198-200, 204-209, 519, 523; CX-0018C (Lebofsky Depo. Tr.) at 154:7-157:22, 160:8-161:18; CX-0019C (Norton Depo. Tr.) at 194:24-195:13. With regard to the scATAC-seq assay, however, gDNA fragments, not mRNA, are barcoded and the assay does not form barcoded polynucleotides through reverse transcription. *See, e.g.,* JX-0091C.00020; CX-1491C.00018.

For the foregoing reasons, I find that WTA 3′ v1, [redacted] infringe claim 4. I further find that the scATAC-seq assay does not infringe claim 4.

b. Claim 11

Claim 11 depends directly from claim 1 and requires that the barcode molecules be released from the gel bead. '530 patent, col. 49:34-36. As discussed above, in the WTA 3′ v1, [redacted] and scATAC-seq assays, [redacted] the barcode molecules

from the gel bead. *See supra.* Accordingly, I find that the accused assays infringe claim 11.

c. Claim 14

Claim 14 requires that there be at least 1,000 barcode molecules "disposed within" each gel bead. '530 patent, col. 49:44-45. The gel beads used in the accused products are formed from polyacrylamide, which is a polymer hydrogel formed by polymerization of acrylamide monomers, acrydite oligos and crosslinker methylene-bis-acrylamide in water. CX-0004C (Butte DWS) at Q/A 116-17, 122-23; JX-0101C.00006. Each gel bead is porous having a three-dimensional network of pores. *Id.* The gel beads are created by combining the acrylamide pre-mix and barcode molecules, which results in barcode molecules bonded throughout each gel bead. CX-1548C.00006. Each resulting bead has at least 1,000,000 barcode molecules disposed within the bead. CX-0004C (Butte DWS) at Q/A 128-31, 133, 546-47; JX-0101C.00006 ("Entire volume is accessible."). Accordingly, I find that the WTA 3′ v1, [redacted], and scATAC-seq assays infringe claim 14.

d. Claim 19

Claim 19 depends from claim 1 through unasserted claim 17. Claim 17 requires that the barcode molecules "comprise combinatorial assemblies of sequences from sequence modules." *Id.*, col. 50:5-7. Claim 19 further requires that the combinatorial assemblies have a first sequence, a second sequence, and a third sequence. *Id.*, col. 50:13-15. The barcode molecules in the accused products comprise [redacted]. JX-0105C.00021; JX-0075C.00018; CX-0004C (Butte DWS) at Q/A 550-51; JX-0101C.0007-.00008. Accordingly, I find that the WTA 3′ v1, [redacted], and scATAC-seq assays infringe claim 19.

e. Claim 26

Claim 26 depends directly from claim 1 and requires that the gel beads have at least 1,000,000 barcode molecules. '530 patent, col. 50:30-31. The gel beads in the accused assays have over 1,000,000 barcode molecules. CX-0004C (Butte DWS) at Q/A 128-29; JX-0050C.00026; JX-0105C.00020-.00022. Accordingly, I find that the WTA 3′ v1, [redacted], and scATAC-seq assays infringe claim 26.

f. Claim 28

Claim 28 depends directly from claim 1 and requires that the barcode molecules be released from the gel bead before the formation of the barcoded polynucleotide molecules. '530 patent, col. 50:35-37. In the accused assays, the [redacted] severs the barcode molecules from the gel bead before the generation of barcoded cDNA strands and barcoded gDNA fragments. CX-0004C (Butte DWS) at Q/A 557-559; *see, e.g.*, JX-0075C.00018. Accordingly, I find that the WTA 3′ v1, [redacted], and scATAC-seq assays infringe claim 28.

3. Indirect Infringement

10X alleges that Bio-Rad indirectly infringed the asserted claims by inducing infringement or through contributory infringement.

a. Underlying Acts of Direct Infringement

Both induced infringement and contributory infringement require an act of direct infringement. *Carborundum Co. v. Molten Metal Equip. Innovations, Inc.*, 72 F.3d 872, 876 n.4 (Fed. Cir. 1995) ("Absent direct infringement of the claims of a patent, there can be neither contributory infringement nor inducement of infringement.") (quoting *Met–Coil Sys. Corp. v. Korners Unlimited, Inc.*, 803 F.2d 684, 687 (Fed. Cir. 1986)) (internal quotation marks omitted). Moreover, the act of direct infringement must be by an entity other than Bio-Rad. *AIDS*

Healthcare Found., Inc. v. Gilead Sci., Inc., 890 F.3d 986, 992-93 (Fed. Cir. 2018) ("Liability for induced infringement requires that some other entity is directly infringing the patent."); *Spansion, Inc. v. Int'l Trade Com'n,* 629 F.3d 1331, 1352 (Fed. Cir. 2010) ("[T]o prevail on contributory infringement in a Section 337 case, the complainant must show: . . . the accused infringer imported, sold for importation, or sold after importation within the United States, the accused components that contributed to another's direct infringement.").

With Bio-Rad's assistance, Berkeley researchers performed SureCell 3' WTA assays, using 12 ddSEQ v1 cartridges. CX-1573.00001. The researchers were able to obtain barcoded cDNA from a total of 18,000 cells resulting in an average of 1500 cells being barcoded per cartridge. *Id.* at .00002-.00003. In addition, [redacted]. CX-1494C.00016. A Bio-Rad document describes another experiment in which a SureCell 3' WTA assay was conducted using a single ddSEQ v1 cartridge. JX-0036.00002-.00003. The experiment resulted in 1,384 cells being barcoded. *Id.* at .00003. 10X, however, has not pointed to any evidence showing that the SureCell 3' WTA assay was used with [redacted]. Accordingly, I find that the SureCell 3' WTA assay has been used with the ddSEQ v1 products to infringe the asserted claims. I further find that 10X has not shown that the SureCell 3' WTA assay has been used with [redacted] to infringe the asserted claims.

Although there is testimony indicating that the [redacted], the testimony does not provide sufficient details that would allow for a determination of whether this was an infringing use. It is possible to use the ddSEQ v1 cartridge in a non-infringing manner by using only a subset of

cartridge's four chambers to conduct an assay. Using only a subset of the chambers will fall outside the scope of the claims because the cartridge will not produce at least 1,000 droplets containing a cell and gel bead. *See, e.g.,* JX-0034.00005 (teaching that, if primed with input cells, each chamber will produce approximately 300 droplets with one cell and one gel bead), .00017 (providing instructions on how to use cartridge without priming all of the chambers with cells). At least one or two of Bio-Rad's customers have so used the ddSEQ v1 cartridge. CX-0016C (Kaihara Depo. Tr.) at 167:18-168:3. There is no evidence regarding the methodology employed with running the [redacted]

With regard to the scATAC-seq assay, in September 19, 2018, Bio-Rad made the scATAC-seq assay available to its "Early Access Customers" for use with the ddSEQ v1 system. CX-1739C; *see also,* CX-0004 (Butte DWS) at Q/A 95. There is, however, no evidence of any of the "Early Access Customers" purchasing, much less using, the scATAC-seq assay for use with the ddSEQ v1 system. As discussed above, although the scATAC-seq [redacted]. Tr. (Kaihara) at 275:2-6; CX-0016C (Kaihara Depo. Tr.) at 148:13-19. Accordingly, I find that 10X has not shown that the scATAC-seq assay has been used with [redacted] the ddSEQ

v1 system [redacted] system to infringe the asserted claims of the '530 patent.[16]

[redacted]

Based on the foregoing, the only acts of direct infringement by entities other than Bio-Rad involve the 3' WTA v1 assay used with the ddSEQ v1 system.

b. Induced Infringement

For induced infringement, 10X must show that Bio-Rad acted to induce infringement of the asserted claims and that it was aware "that the induced acts constitute[d] patent infringement." *Global-Tech,* 563 U.S. at 760-66. As confirmed by the testimony of Bio-Rad witnesses, Bio-Rad actively induced end-users to infringe the asserted claims by using the 3' WTA v1 assay with the ddSEQ v1 system. *See, e.g.,* CX-0019C (Norton Depo. Tr.) at 32:6-11 (testifying that Bio-Rad will "generally train the customer after they purchase the system"), 32:15-33:4 (testifying that Bio-Rad demonstrated the ddSEQ v1 system to each of its customers and that the demonstrations taught the customers "each step of the workflow to use the ddSEQ system"). Bio-Rad provides customers with specific instructions on how to perform the 3' WTA v1 by priming all four chambers of the ddSEQ v1 cartridge with cells. JX-0034.00017 ("To load the same cell sample across all 4 chambers, make a Cell Suspension Mix using the volumes listed for 1 cartridge."). As discussed above, if all four chambers are primed with cells, the

16 [redacted]. Bio-Rad's own acts of direct infringement, however, cannot be relied upon to support a finding of indirect infringement. *AIDS Healthcare,* 890 F.3d at 992-93; *Spansion,* 629 F.3d at 1352.

cartridge will generate approximately 1,200 droplets containing a cell and gel bead.

With regard to Bio-Rad's knowledge that the induced acts constituted patent infringement, Bio-Rad was aware of the 10X's infringement allegations and "the '530 Patent as of at least January 15, 2018, when 10X served its summons and complaint in *10X Genomics, Inc. v. Bio-Rad Labs., Inc.*, Case No. 3:18-cv-00209 (N.D. Cal. Jan. 9, 2018)." Bio-Rad's Response to the Complaint (Mar. 6, 2018), ¶ 86. With regard to whether Bio-Rad knew that the acts that induced were intended to cause a third party to infringe the '530 patent, there is no evidence that Bio-Rad sought and obtained a non-infringement opinion. Bio-Rad's failure to do so is circumstantial evidence that it was aware that the acts brought about by its conduct would infringe the '530 patent. *See, e.g., Broadcom Corp. v. Qualcomm Inc.*, 543 F.3d 683, 699 (Fed. Cir. 2008) (failure to procure a non-infringement opinion is "circumstantial evidence of intent to infringe").

Based on the foregoing, I find that Bio-Rad induced infringement of the asserted claims of the '530 patent by inducing others to use the 3' WTA v1 assay with the ddSEQ v1 system.

c. Contributory Infringement

"[T]o prevail on contributory infringement in a Section 337 case, the complainant must show *inter alia*: (1) there is an act of direct infringement in violation of Section 337; (2) the accused device has no substantial non-infringing uses; and (3) the accused infringer imported, sold for importation, or sold after importation within the United States, the accused components that contributed to another's direct infringement." *Spansion,* 629 F.3d at 1353. With regard to the first and third elements, as discussed above, Bio-Rad "imported, sold for importation, or sold after importation within the United States" the 3' WTA v1 assay and the ddSEQ v1 products, which were used by others to infringe the asserted claims of the '530 patent.

With regard to the second element identified by the *Spansion* court, it is 10X's burden to show "that there are no substantial non-infringing uses" of the accused system. *Toshiba Corp. v. Imation Corp.*, 681 F.3d 1358, 1362 (Fed. Cir. 2012) (citing 35 U.S.C. § 271(c)). Pointing to three uses for the accused product that it contends are substantial and non-infringing, Bio-Rad argues that 10X has not met its burden.[17] "[N]on-infringing uses are substantial when they are not unusual, far-fetched, illusory, impractical, occasional, aberrant, or experimental." *Id.* (quoting *Vita–Mix Corp. v. Basic Holding, Inc.*, 581 F.3d 1317, 1327 (Fed. Cir. 2009) (internal quotation marks omitted). "In assessing whether a use is substantial, the fact-finder may consider 'the use's frequency, . . . the use's practicality, the invention's intended purpose, and the intended market.'" *Id.* (quoting *i4i Ltd. P'ship v. Microsoft Corp.*, 598 F.3d 831, 851 (Fed. Cir. 2010)) (omission in original).

The first use that Bio-Rad contends is substantial and non-infringing is the DROP-seq assay. This assay and Bio-Rad's contentions are addressed above. I find that the DROP-seq assay is not a substantial, non-infringing use of the accused ddSEQ v1 products with respect to the asserted claims of the '530 patent for the same reasons that it does not constitute such a use with the respect to the '024 patent.

The second alleged substantial non-infringing use is processing samples using less than all four chambers of the ddSEQ v1 cartridge. The asserted claims require the generation of at least 1,000 droplets containing a single cell and a single gel bead. '530 patent, col. 48:65-col.

[17] Staff—but not Bio-Rad—identifies a fourth alleged non-infringing use: [redacted]

49:2 (claim 1). The ddSEQ v1 cartridge has four chambers and each chamber is capable of producing an average of 300 droplets containing a cell and gel bead. JX-0034.0005. Accordingly, if less than four of the chambers are used, the ddSEQ v1 cartridge will generate less than 1,000 droplets containing a cell and a gel bead. RX-0665 (Metzker DWS) at Q/A 148. Bio-Rad provides instructions on how to use the cartridge without using all four chambers to process samples:

> All 4 sample chambers must be loaded with Cell Suspension Mix. If you choose not to bad any cells into a chamber, prepare and load the Cell Suspension Mix, substituting an equivalent volume 1X PBS +0.1% BSA in place of Filtered Cells.

JX-0034.00017.

Although it is possible to use the ddSEQ v1 cartridge without using all four chambers, the evidence indicates that such usage would be an uncommon practice at best. In order to generate 1,200 droplets containing a cell and gel bead, Bio-Rad teaches that each of the four chambers should be loaded with between 10,125-12,375 cells. JX-0034.00012. It is Bio-Rad's expectation that its customers will use all four chambers and it counsels potential customers with less than 40,000 cells that the ddSEQ v1 "system is not right for them." JX-0016C (Kaihara Depo. Tr.) at 167:5-17. [redacted] *Id.* at 167:18-22. [redacted] Accordingly, I find that using less than all four chambers of the ddSEQ v1 cartridge for an assay is not a substantial use of the ddSEQ v1 cartridge.

The third alleged substantial non-infringing use is performing the scATAC-seq assay using purified nuclei, instead of cells. While the scATAC-seq assay can be used to generate droplets containing a cell and gel bead, it can also be used to generate droplets containing purified nuclei and a gel bead. CX-0004C (Butte DWS) at Q/A 92 ("The scATAC-seq assay can

partition either whole cells or purified nuclei for analysis."). Using the assay to encapsulate nuclei instead of cells is a non-infringing use because the claims require the generation of droplets containing a cell and gel bead. '530 patent, col. 48:59-63.

10X does not dispute that using the assay to encapsulate purified nuclei is a non-infringing use, but takes the position that "[t]here is no substantial use of scATAC-seq with isolated nuclei." CIB at 233. In support of its position, 10X argues that "using isolated nuclei rather than single cells is merely an option for scATAC-seq" and that "all use of scATAC-seq is at most [redacted]." *Id.* 10X's argument is unpersuasive. Although Bio-Rad has not fully released the scATAC-seq assay, on September 19, 2018 Bio-Rad started to offer the assay to "Early Access Customers" for use with the ddSEQ v1 system. CX-1739C; CX-0004 (Butte DWS) at Q/A 95. Although using the assay with nuclei instead of cells may only be an "option," it is an option customers will likely select in particular situations. Bio-Rad developed alternate protocols for the scATAC-seq assay—one using cells and one using nuclei—because for certain types of cells "one would work better than the other." CX-0018C (Lebofsky Depo. Tr.) at 157:24-158:16. End users would be expected to use nuclei with the scATAC-seq assay in those instances where using nuclei "would work better" than using intact cells and *vice versa*.

Based on the foregoing, I find that 10X has failed to show that using the scATAC-seq assay with isolated nuclei is not a substantial non-infringing use of the ddSEQ v1 products.[18]

[18] Pointing to the hearing testimony of Dr. Kaihara, Staff argues that using the scATAC assay with nuclei is not a substantial non-infringing use of the ddSEQ v1 products because "[t]he evidence shows that ATAC-seq on the v1 products, [redacted] SIB at 105. Although Staff correctly characterizes Dr. Kaihara's testimony, it ignores that Bio-Rad offered the scATAC assay to its customers for use with the ddSEQ v1 products. CX-1739C; CX-0004 (Butte DWS) at Q/A 95.

D. Domestic Industry

10X asserts that its single-cell domestic industry products practice claims 1, 4, 11, 14, 19, 26, and 28 of the '530 patent.

1. Claim 1

a. Preamble

To the extent that the preamble is limiting, there is no dispute that the domestic industry products either are methods of nucleic acid preparation and analysis or are used in such methods. '530 patent, col. 47:58-59. Specifically, 10X's single cell applications are used to prepare cell samples for transcriptome analysis. CX-0004C (Butte DWS) at Q/A 564.

b. Step 1: "providing" a plurality of cells and at least 1,000 gel beads

There is no dispute that the domestic industry products provide at least 1,000 gel beads and a plurality of cells. *See, e.g.,* CX-0477.00001 ("Within each microfluidic channel, ~100,000 GEMs are formed per ~6-min run, encapsulating thousands of cells in GEMs."), .00002 ("The core of the technology is a Gel bead in EMulsion (GEM). GEM generation takes place in an 8-channel microfluidic chip that encapsulates single gel beads at ~80% fill rate . . . ."); CX-0004C (Butte DWS) at Q/A 566. Each cell contains a plurality of polynucleotides in the form of mRNA. CX-0004C (Butte DWS) at Q/A 260, 570; CX-0481.00015; CX-0477.00004. Each gel bead has millions of barcode molecules attached to it. CX-0447.00002 ("Each gel bead is functionalized with barcoded oligonucleotides that consists of: (i) sequencing adapters and primers, (ii) a 14 bp barcode drawn from ~750,000 designed sequences to index GEMs, (iii) a 10 bp randomer to index molecules (unique molecular identifier, UMI) and (iv) an anchored 30 bp oligo-dT to prime polyadenylated RNA transcripts . . . ."); CX-0004C (Butte DWS) at Q/A 263. The barcode molecules comprise identical barcode sequences that are distinct from the barcode

sequences of the barcode molecules attached to any other gel bead. *Id.* The barcode molecules are releasably attached to the gel beads through a [redacted] that can be broken through the application of [redacted]. CX-0477.00002 ("Gel beads dissolve and release their oligonucleotides for reverse transcription of polyadenylated RNAs."); CX-0004C (Butte DWS) at Q/A 266.

Based on the foregoing, I find that the single-cell domestic industry products satisfy the first step of claim 1.

c. Step 2: "generating a plurality of droplets"

As required by the second step of claim 1, the domestic industry products generate a plurality of droplets, wherein at least 1,000 of the droplets comprise a "single gel bead" and a "single cell." *See, e.g.,* CX-0477.00001 ("Within each microfluidic channel, ~100,000 GEMs are formed per ~6-min run, encapsulating thousands of cells in GEMs."), .00002 ("The core of the technology is a Gel bead in EMulsion (GEM). GEM generation takes place in an 8-channel microfluidic chip that encapsulates single gel beads at ~80% fill rate . . . ."); CX-0004C (Butte DWS) at Q/A 566.

Bio-Rad argues that the domestic industry products do not satisfy the second step of claim 1 because the products do not "generate a collection of 'at least 1,000 droplets' each having a 'single gel bead' and 'single cell.'" RIB at 198. This is the same argument that Bio-Rad made with respect to the accused products: Because the cells start to lyse almost immediately after droplet formation, at any instant of time there are less than 1,000 droplets with a cell and gel bead. *Id.* As discussed above in the context of infringement, claim 1 only requires the generation of at least 1,000 droplets containing a cell and gel bead before the third step, not that a "collection" of such droplets exist before the start of the third step.

Based on the foregoing, I find that the accused products satisfy the second step of claim 1.

d. Step 3: generating a plurality of "barcoded polynucleotide molecules"

The domestic industry products perform the third step of claim 1 when the droplets are heated on the thermal cycler. After the droplets are generated, they are transferred to a thermal cycler and heated at 53°C for 45 minutes and then heated at 85°C for 5 minutes. CX-0481.00013. While the droplets are being heated on the thermal cycler, the barcode molecules are released from the gel bead through the application of [redacted], which dissolves [redacted] holding the barcode molecules to the gel beads. CX-0481.00011; CX-0004C (Butte DWS) at Q/A 481. In each of at least 1,000 droplets, two or more barcoded polynucleotide molecules are generated using the mRNA from the cell and the barcode molecules from the bead. CX-0481.00011 ("Incubation of the GEMs then produces barcoded, full-length cDNA from poly-adenylated mRNA."); CX-0004C (Butte DWS) at Q/A 576-78.

Bio-Rad argues that 10X has "not provided any evidence showing that a plurality of barcoded polynucleotides are formed in each droplet on 10X's thermocycler." RIB at 199. Bio-Rad's argument is the same as the one it made with respect to accused products: Because cell lysis begins as soon as the droplets are formed, the generation of barcoded polynucleotides begins before the droplets are incubated. This argument fails for the same reasons that it failed in the context of infringement.

According to 10X documents, barcoded polynucleotides are generated when the droplets are being heated on the thermal cycler. CX-0481.00011 ("Incubation of the GEMs then produces barcoded, full-length cDNA from poly-adenylated mRNA."); *see also* CX-0004C (Butte DWS) at Q/A 576-78. To counter this evidence, Bio-Rad points to the testimony of its

expert Dr. Metzker, who testifies that the generation of barcoded mRNA starts in each droplet almost as soon as the droplet is formed. RX-0665C (Metzker RWS) at Q/A 117. Dr. Metzker, however, does not testify that barcoding is completed in any or all of the droplets before they are incubated. Even if barcoded polynucleotides start to form immediately after the droplet is formed, there is no evidence that the generation of barcoded polynucleotides would be completed in any of the droplets before they are transferred to the thermal cycler.

The droplets in the domestic industry product are heated at temperatures and durations similar to those used in the accused products to stimulate the release of the barcode molecules from the gel beads and the generation of the barcoded molecules. In the domestic industry products, droplets are generated at room temperature (~20°C) in 6.5 minutes. CX-0481.00013 ("GEM Generation – 6.5 minutes"), .00018 ("Equilibrate to room temperature before use . . . ."). In the ddSEQ v1 products, the droplets are generated at room temperature in five minutes. JX-0034.00005. In the domestic industry products, the droplets are heated on the thermal cycler at 53°C for 45 minutes and then at 85°C for 5 minutes. CX-0481.00026. In the ddSEQ v1 products, the droplets are heated ion the thermal cycler at 37°C for 30 minutes and then at 50°C for 60 minutes. JX0034.00026. Similar to 10X's documentation for the domestic industry products, Bio-Rad's documentation describes barcoded cDNA being generated through reverse transcription while the droplets are being heated on the thermal cycler. *See, e.g.,* JX-0088C.00015; JX-0034.00043; JX-0034C.00025, .00026.

Based on the foregoing, I find that the domestic industry products practice claim 1.

2. Claims 4, 11, 14, 19, 26, and 28

Claim 4, 11, 14, 19, 26, and 28 depend either directly or indirectly from claim 1. There is no dispute that the single-cell domestic industry products satisfy the additional limitations of these dependent claims.

a. Claim 4

The cells provided by the domestic industry products have mRNA. CX-0004C (Butte DWS) at Q/A 260, 570; CX-0481.00015; CX-0477.00004. As further required by claim 4, barcoded polynucleotides are generated by reverse transcribing the mRNA in the presence of the barcode molecules. CX-0481.00011; CX-0004C (Butte DWS) at Q/A 576-78. Accordingly, I find that the domestic industry products practice claim 4.

b. Claim 11

As required by claim 11, in each droplet, the barcode molecules are released from the gel bead. CX-0481.00011; CX-0004C (Butte DWS) at Q/A 481. Accordingly, I find that the domestic industry products practice claim 11.

c. Claim 14

In accordance with claim 14, there at least 1,000 barcode molecules "disposed within" each gel bead. Specifically, the beads are porous polyacrylamide gel beads. CX-0004C (Butte DWS) at Q/A 587. Each gel bead has over 1,000 barcode molecules disposed throughout its entire volume. *Id.*; CX-0479C.00010; CX-0542.00001 ("Each Gel Bead contains millions of oligo primers . . . ."). Accordingly, I find that the domestic industry products practice claim 14.

d. Claim 19

The barcode molecules of the domestic industry products include combinatorial assemblies of sequences formed from sequence modules. CX-0004C (Butte DWS) at Q/A 589; CX-0425C.00016; JX-0037C.00036-.00042. As required by claim 19, each of the combinatorial

assemblies has a first sequence [redacted] a second sequence [redacted] and a third sequence [redacted] *Id.* Accordingly, I find that the domestic industry products practice claim 19.

e. Claim 26

As required by claim 26, the gel beads have at least 1,000,000 barcode molecules. CX-0481.00061 ("Gel Beads are the foundation of 10x Genomics®' technology, and are beads functionalized with millions of copies of a 10x Barcoded primer."); CX-0004C (Butte DWS) at Q/A 263, 592. Accordingly, I find that the domestic industry products practice claim 26.

f. Claim 28

As required by claim 28, the barcode molecules of the domestic industry products become detached from the gel beads before the barcoded polynucleotide molecules are generated. CX-0004C (Butte DWS) at Q/A 275, 594; CX-0542.00001 ("Once partitioned, the Gel Bead dissolves and its oligo primers are released into the aqueous environment of the GEM. The cell captured in the GEM is also lysed. The contents of the GEM (oligos, lysed cell components and Master Mix) are incubated in an RT reaction to generate full-length, barcoded cDNA from the poly A-tailed mRNA transcripts."). Accordingly, I find that the domestic industry products practice claim 28.

E. Invalidity

Bio-Rad contends that the asserted claims of the '530 patent are invalid as anticipated or rendered obvious by the '059 patent and/or the Church patent, alone or in combination with additional prior art. RIB at 199-215. All of the recited claims require barcode molecules that are "releasably attached" to a gel bead, and the parties' arguments regarding invalidity for the '530 patent are substantially identical to those addressed above in the context of the "releasable

attachment" limitation of the '024 patent. *See* SRB at 107. For the same reasons discussed above, Bio-Rad has failed to show that any asserted claim of the '530 patent is anticipated and/or rendered obvious by the '059 patent and/or the Church patent because these references do not disclose the "releasably attached" limitation.[19] In addition, the success of 10X's domestic industry products further weigh against a finding of obviousness.

VIII. ADDITIONAL DEFENSES

A. Inventorship

1. Pertinent Factual Background

In 2008, Dr. Benjamin Hindson and others founded QuantaLife to develop a droplet digital PCR system. Tr. (Hindson) 137:20-22; CX-0001C (Hindson WS) at Q/A 22-25. Dr. Hindson was Chief Scientific Officer. Tr. (Hindson) at 137:23-25; CX-0001C (Hindson WS) at Q/A 25. Dr. Nicholas Heredia joined QuantaLife in May 2009 as a Senior Molecular Biologist. RX-504C (Heredia WS) at Q/A 8. Dr. Serge Saxonov joined QuantaLife in 2010. Tr. (Saxonov) at 771:13-15. Dr. Saxonov was Vice President of Application Development for QuantaLife's droplet digital PCR system. *Id.* at 771:16-21.

In 2011, Bio-Rad purchased QuantaLife. CX-0001C (Hindson WS) at Q/A 31. Drs. Hindson, Saxonov, and Heredia became Bio-Rad employees. Tr. (Saxonov) at 771:9-12); CX-

[19] 10X further contends that the '059 patent and the Church patent fail to disclose the step of "generating" droplets, CIB at 241-42, arguing for a distinction between the term "merging" and the term "generating," which is supported by Dr. Dear's citation to deposition testimony from Dr. Agresti. *See* CX-1827C (Dear RWS) at Q/A 604-09, 719-22. 10X cites no evidence from the intrinsic record that the claim language of the '530 patent makes a distinction between "merging" and "generating" droplets, however. The '059 patent includes several paragraphs under the heading "Droplet Generation," which includes discussions of emulsions and the coalescence of smaller droplets with larger droplets. JX-0031, col. 13:5-37. In addition, Dr. Metzker has identified specific disclosures in the '059 patent and in Church that meet this limitation. RX-0664C at Q/A 349, 382.

0001C (Hindson WS) at Q/A 33; RX-0504C (Heredia WS) at Q/A 6. Dr. Hindson's role at Bio-Rad [redacted] CX-0001C (Hindson WS) at Q/A34. In April 2012, Drs. Hindson and Saxonov resigned their positions at Bio-Rad on the same day. Tr. (Hindson) at 163:6-14. Dr. Heredia remained at Bio-Rad, where he still works. RX-0504C (Heredia WS) at Q/A 3, 6. Three months later, after taking a break from work, Drs. Hindson and Saxonov founded 10X. Tr. (Hindson) at 163:22-24; CX-0001C (Hindson WS) at Q/A 38-40. The first provisional patent applications for the asserted patents were filed in August 2012. *See, e.g.,* JX-0003 ('024 patent, cover), JX-0005 ('468 patent, cover).

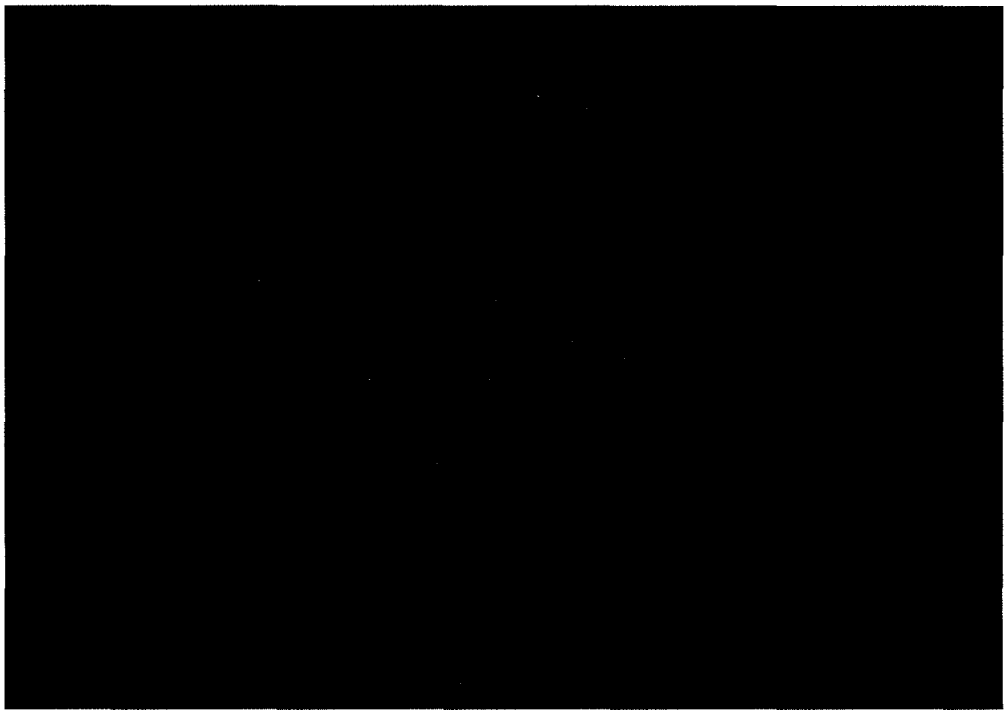
Bio-Rad and Dr. Heredia claim that Dr. Heredia was improperly omitted as a co-inventor on the asserted patents, and that the patents are therefore invalid. *Pannu v. Iolab Corp.,* 155 F.3d 1344, 1349 (Fed. Cir. 1998) ("[I]f nonjoinder of an actual inventor is proved by clear and convincing evidence . . . a patent is rendered invalid."). Bio-Rad contends that Drs. Hindson and Saxonov, while they were at QuantaLife, "built off of" the "fundamental solution that Hindson and Heredia had come up with [redacted]." Tr. (Opening Statement) at 95:8-10. 10X counters that the technology described in the asserted patents is distinct from anything that was described by Dr. Heredia or worked on by him or any others at QuantaLife.

2. Alleged inventorship

Dr. Heredia claims to be an inventor on all four of the patents in suit. RX-0504C (Heredia WS) at Q/A 20 [redacted]

[redacted]

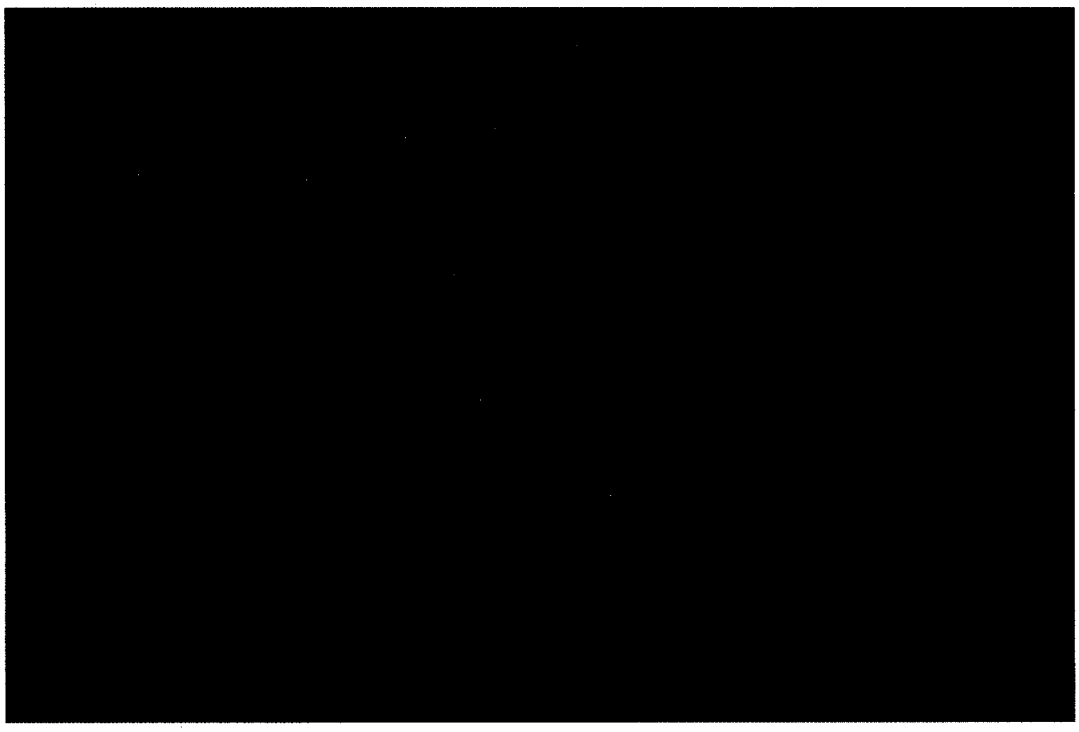
In terms of his claim of co-inventorship, however, he points only to his work with Dr. Hindson. *Id.* at Q/A 11. Dr. Heredia alludes to [redacted]



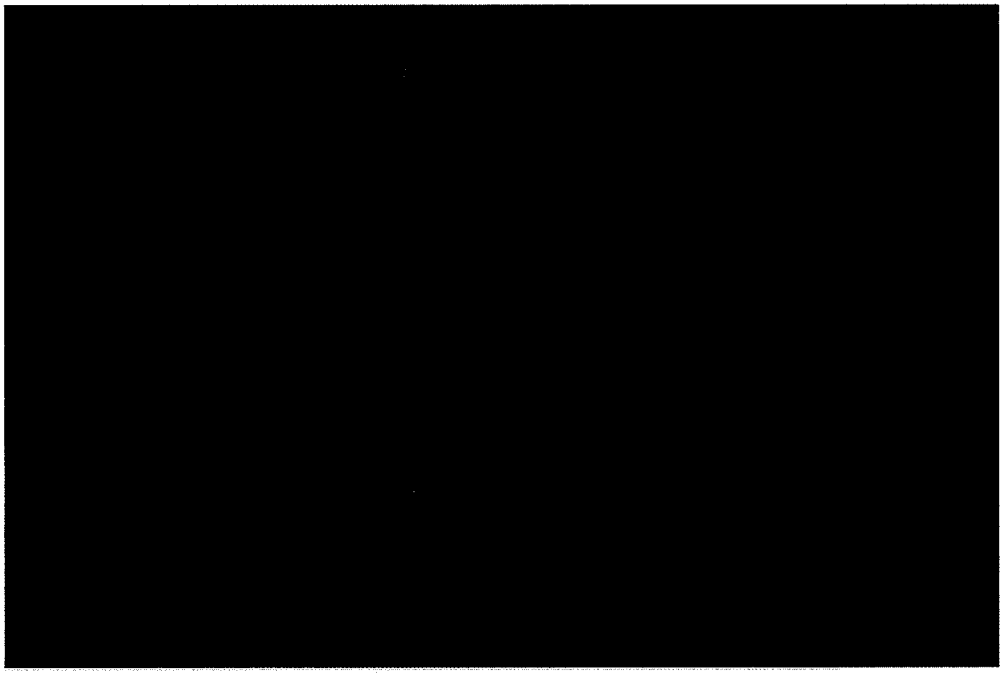
JX-0057C.00018 .

[redacted]

[redacted][20]



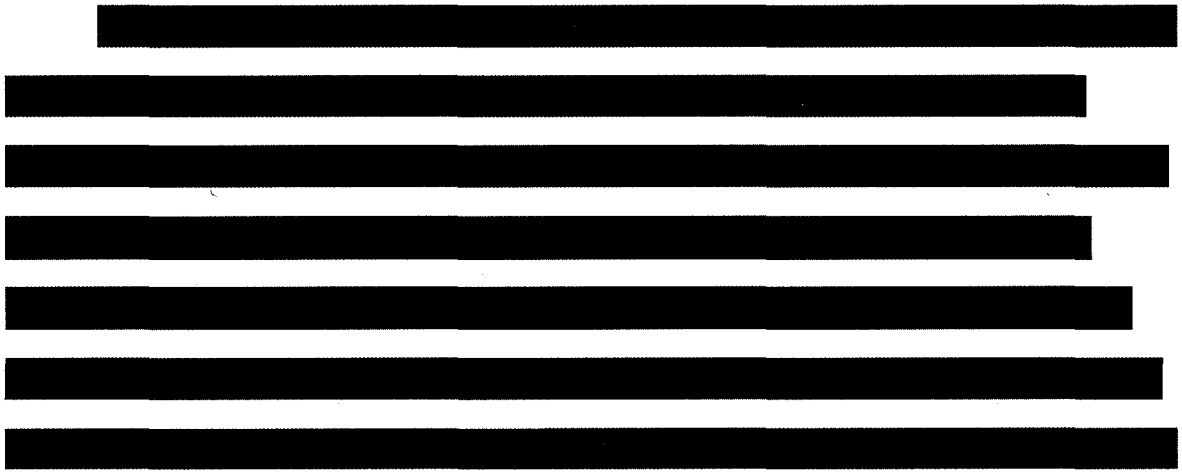
JX-0120C.00007;



JX-120C.00008; and

PUBLIC VERSION

JX-0120C.00009



[redacted]

3. Discussion

a. Legal Standards

The statutory requirements regarding joint inventorship state, in pertinent part:

> When an invention is made by two or more persons jointly, they shall apply for patent jointly and each make the required oath, except as otherwise provided in this title. Inventors may apply for a patent jointly even though (1) they did not physically work together or at the same time, (2) each did not make the same type or amount of contribution, or (3) each did not make a contribution to the subject matter of every claim of the patent.

35 U.S.C. § 116 (a).

A joint invention is "the product of collaboration," and requires that "each of the inventors work on the same subject matter and make some contribution to the inventive thought and to the final result.'" *Vanderbilt Univ. v. ICOS Corp.*, 601 F.3d 1297, 1302 (Fed. Cir. 2010) (quoting *Monsanto Co. v. Kamp,* 269 F. Supp. 818, 824 (D.D.C. 1967)) (internal quotation marks omitted). "[T]he critical question for joint conception is who conceived, as that term is used in the patent law, the subject matter of the claims at issue." *Ethicon, Inc. v. U.S. Surgical Corp.,* 135 F.3d 1456, 1460 (Fed. Cir. 1998). "Conception is the touchstone of inventorship" and "[i]t is 'the formation in the mind of the inventor, of a definite and permanent idea of the complete and operative invention, as it is hereafter to be applied in practice.'" *Burroughs Wellcome Co. v.*

---

[21] In several instances, Dr. Heredia's testimony at hearing had evolved significantly from the testimony he gave at his deposition. For example, with respect to the issue discussed above, whether [redacted] CX-0014C at 211:14-21. This was the pattern of Dr. Heredia's testimony throughout the hearing. *See infra.* His credibility suffers as a result.

Barr Labs. Inc., 40 F.3d 1223, 1227-28 (Fed. Cir. 1994) (quoting *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1376 (Fed. Cir. 1986)).

"It is not necessary that the entire invention concept should occur to each of the joint inventors . . . ." *Vanderbilt,* 601 F.3d at 1302 (quoting *Monsanto,* 269 F. Supp. at 824). "'[E]ach contributor need not have their own contemporaneous picture of the final claimed invention in order to qualify as joint inventors.'" *Id.* at 1303 (citing *Fina Oil & Chem. Co. v. Ewen,* 123 F.3d 1466, 1473 (Fed. Cir. 1997)). However, "'[o]ne who simply provides the inventor with well-known principles or explains the state of the art without ever having a firm and definite idea of the claimed combination as a whole does not qualify as a joint inventor.'" *Nartron Corp. v. Schukra U.S.A. Inc.,* 558 F.3d 1352, 1356 (Fed. Cir. 2009) (quoting *Ethicon,* 135 F.3d at 1460). "'[T]he qualitative contribution of each collaborator is the key—each inventor must contribute to the joint arrival at a definite and permanent idea of the invention as it will be used in practice.'" *Vanderbilt,* 601 F.3d at 1303 (quoting *Burroughs,* 40 F.3d at 1229).

In general, "'[t]he inventors as named in an issued patent are presumed to be correct.'" *Nartron,* 558 F.3d at 1356 (quoting *Hess v. Advanced Cardiovascular Sys., Inc.,* 106 F.3d 976, 980 (Fed. Cir. 1997). Proof of joint inventorship requires clear and convincing evidence. *Vanderbilt,* 601 F.3d at 1305.

b. Insufficient evidence of collaboration

"A primary focus of section 116 has [] always been on collaboration and joint behavior." *Vanderbilt,* 601 F.3d at 1303. "The interplay between conception and collaboration requires that each co-inventor engage with the other co-inventors to contribute to a joint conception." *Id.*

Dr. Hindson testifies that he did not collaborate with Dr. Heredia on the [redacted]. He explains that Dr. Heredia was a new employee at QuantaLife in May 2009 and that his role

was limited to "assisting in validation and testing." CX-1828C (Hindson RWS) at Q/A 6; *see* Tr. at 181:19-23 [redacted] He recalls discussing Dr. Heredia's [redacted] for five or 10 minutes, but he states that [redacted] CX-1828C (Hindson RWS) at Q/A 11; Tr. at 181:24-182:16. Dr. Hindson says he commented to Dr. Heredia that he had done well to put his idea down on paper only because he did not want to discourage him. CX-1828C at Q/A 11. Dr. Hindson testifies that he did not know of any follow-up work on Dr. Heredia's [redacted], that no research plan was developed based on the idea, that no experiments were conducted, and that the idea did not inform any work Dr. Hindson performed at QuantaLife or Bio-Rad. *Id.* at Q/A 21-22.

Dr. Heredia does not specifically dispute Dr. Hindson's recollection that there was no significant collaboration between him and Dr. Hindson based on the [redacted]. *See, e.g.,* CX-0015C (Heredia Dep.) at 335:21-336:2 ([redacted]). He maintains, however, that [redacted] show "an inventive contribution," Tr. (Heredia) at 602:7-13, and collaboration, and that [redacted], JX-0120C, shows follow-up. RX-0504C at Q/A 16 [redacted] He recalls no subsequent development or any additional conversations with Dr. Hindson or others at QuantaLife concerning [redacted], however. *E.g.,* Tr. (Heredia) at 584:15-19 (". . . I can't recall . . . . But I have a vague sense that Dr. Hindson talked about, you know, [redacted]

[redacted] 594:12-18 ([redacted]. Dr. Heredia also testifies that he recalls no conversations with Dr. Saxonov about the [redacted]. *Id.* at 594:7-11.

In sum, apart from Dr. Heredia's somewhat vague and uncertain testimony, his [redacted], which do not clearly demonstrate a collaborative effort to develop Dr. Heredia's idea, Bio-Rad points to no evidence to show that Dr. Heredia collaborated with other scientists at QuantaLife or Bio-Rad on any project concerning the [redacted]. Dr. Hindson denies that such a collaboration occurred, and Dr. Heredia cannot recall any specific collaborative activities concerning development of his [redacted], beyond the alleged brainstorming discussion with Dr. Hindson, the details of which are disputed by Dr. Hindson. On this record, I find insufficient evidence to establish that Dr. Heredia collaborated with others to develop the technology in the asserted patents.

c. Insufficient evidence of conception

The "core idea" of the "gel bead-in-emulsion" or "GEM" architecture claimed in the asserted patents

> is about partitioning nucleic acids, DNA or RNA, in droplets together with gel beads that are used to deliver the barcodes into the droplet. The gel beads contain oligonucleotide barcodes. In each gel bead there are a large number of oligonucleotide molecules that include barcode sequences . . . Those oligonucleatide barcodes are released from the gel beads using a stimulus. They attach to the nucleic acids in the droplet. An amplification reaction is used to create barcoded nucleic acids, and those can be used for downstream processing.

CX-0003C (Schnall-Levin WS) at Q/A 27 (discussing the '024 patent).

Dr. Heredia's [redacted] *Id.* at .00004. [redacted] *Id.* at .00005.

Bio-Rad's argument is that Dr. Heredia's [redacted] address the same problem of "sample preparation for analysis of biological materials such as nucleic acids," that ultimately is addressed in the asserted patents. Tr. at 86: 22-23. Bio-Rad maintains that Dr. Heredia's solution to the problem can be reduced to four parts that track the invention described in the asserted patents: First, Bio Rad identifies "partitioning the sample into droplets." *Id.* at 86:25-87:1. Second is "creating a reagent delivery system." *Id.* at 87:1-2. Third is "combining the sample and reagent delivery system with droplets using microfluidics." *Id.* at 87:2-3. Fourth is tracking "the sample reagent reaction

complex with a barcode mechanism." *Id.* at 87:4-5. Bio-Rad's counsel use this construct to maintain that all of the elements ultimately found in the patents-in-issue were conceptualized by Dr. Heredia in 2009.

The evidence fails to persuade clearly and convincingly that Dr. Heredia in 2009 had in mind anything like the architecture of the GEM, however, as described by Dr. Schnall-Levin, above. This is true for several reasons.

Fundamentally, nothing in Dr. Heredia's materials indicates how his idea would work. Dr. Heredia's [redacted] does not explain even on a basic level how his [redacted] functions. CX-1827C (Dear RWS) at Q/A 1145 ("The description in [redacted] is a bare sketch of at best a partially formed idea that does not show any way to deal with even the basic issues that would confront someone trying to make such a thing work."); *see* CX-1828C (Hindson RWS) at Q/A 16-19. One who "merely suggests an idea of a result to be accomplished, rather than means of accomplishing it, is not a joint inventor." *Nartron,* 558 F.3d at 1359 (quoting *Garrett Corp. v. United States,* 422 F.2d 874, 881 (1970)).

More specifically, Bio-Rad's efforts, through Dr. Metzker, to isolate various aspects of the patented technology to claim that they were conceived by Dr. Heredia in 2009 fail due to lack of evidentiary support. For example, Dr. Metzker indicates that Dr. Heredia's inventive contribution was "a reagent delivery system." Tr. (Metzker) at 717:5-22; 716:19-21 ("thinking about it as a reagent delivery system within an aqueous droplet"). Dr. Heredia, however, appears to have had no idea of the reagent delivery system described in the asserted patents. Tr. (Heredia) at 589:6-11 ([redacted]);

Tr. (Heredia) at 590:9-22 ([redacted]).

Bio-Rad seeks to draw an equivalence between the [redacted] described by Dr. Heredia and the porous gel bead described in the asserted patents and used in 10X's GEM technology. The effort fails. First, Dr. Metzker concedes that Dr. Heredia's idea entailed the use of a [redacted] that was "already in the art." Tr. at 718:7-8. He testifies that Dr. Heredia's [redacted] are [redacted] Tr. at 712:19-21. Luminex beads were "extremely well understood at the time of Dr. Heredia's lab notebook entry," Dr. Metzker says. Tr. 716:3-5. Dr. Metzker concedes that "the idea of a capsule in a droplet that can release its contents into the droplet also "might very well be" something known in the state of the art at the time. Tr. at 722:22-723:12. Putting an analyte within an aqueous droplet was "certainly known state of the art by 2009," Dr. Metzker testifies. *Id.* at 724:12-22. The case law is clear that merely describing prior art is not an inventive idea. *Nartron,* 558 F.3d at 1356.

In addition, Dr. Heredia's idea of a [redacted] does not encompass the functionality of a gel bead. Dr. Metzker opines that Dr. Heredia's [redacted] RX-0664C (Metzker DWS at Q/A 480). These assertions are unconvincing. As explained by 10X's expert, Dr. Dear, Dr. Heredia's [redacted] which is not the same as a bead, a distinction that would have been understood in the art at the time. CX-1827C (Dear RWS) at Q/A 1148. [redacted] *Id.* at Q/A 1149. *See* Tr. (Hindson) at

172:6-7 (describing Luminex as "a capture bead, a solid bead that's used to capture analytes from solution for subsequent detection"); *see also* CX-1828C (Hindson RWS) at Q/A 10.

Dr. Heredia not only did not describe the functionality of the patented gel beads in his 2009 materials, he did not conceive of that functionality. As noted by Dr. Dear, Dr. Heredia at deposition and at trial "struggled to articulate an understanding of what the relationship between [redacted] and any of the claimed inventions was." *Id.* at Q/A 1172. In fact, Dr. Heredia apparently struggled at his deposition to understand what a porous gel bead (as disclosed in the asserted patents), actually is. *See, e.g.,* CX-0014C (Heredia Dep.) at 42:2-11 ("Q. [] Are porous gel beads and [redacted] the same thing? A. Well, gels are just an extremely viscous liquid, in my understanding. So they're very related."). Dr. Metzker, Bio-Rad's expert, implicitly contradicts Dr. Heredia's testimony, agreeing that "a gel is not a viscous liquid," *id.* at 713:5, and rejects the notion that Dr. Heredia's [redacted] is a gel. Tr. (Metzker) at 712:23-25, 713:5-8. Although Dr. Metzker testifies that Dr. Heredia's [redacted] Tr. at 712:14-22, he admits that nothing in Dr. Heredia's depiction of his [redacted] indicates that it was either porous or a gel. Tr. 714:10-2. Dr. Heredia himself cannot say whether in 2009 he knew what a porous gel bead was. Tr. 581:18-22.

Dr. Metzker opines that "Dr. Heredia specifically conceived and contributed [redacted] RX-0664C (Metzker DWS) at Q/A 480. Dr. Metzker opines that Dr. Heredia's idea as set forth in [redacted] *Id.* Dr. Metzker says Dr. Heredia's idea [redacted]

[redacted] *Id.* at 481. Dr. Heredia's materials, however, depict no mechanism for achieving this result. As noted above, Dr. Heredia himself testifies that he cannot recall having "the idea of applying a stimulus to a bead to release oligonucleotide molecules from it." Tr. (Heredia) at 590:9-22. Dr. Dear confirms that Dr. Heredia's [redacted] does not depict oligonucleotide barcodes or barcodes releasably attached to anything; it does not show any attachment that is releasable upon the application of a stimulus. CX-1827C (Dear RWS) at Q/A 1142. Dr. Heredia himself concedes that the oligonucleotides he envisioned were "not going to be released into the interior." CX-0014C (Heredia Dep.) at 172:21-173:4; *see also* Tr. at 589:6-11. Again, Dr. Metzker's efforts to extrapolate elements of the GEM architecture from Dr. Heredia's depictions of his liquid [redacted] are unpersuasive.

Even if one were to accept the proposition that Dr. Heredia's [redacted] could be considered a porous gel bead, Dr. Heredia's notebook does not disclose barcoding nucleic acids or any microfluidic system; it does not disclose a barcode that can function as a unique label; and it does not disclose what the numbers of droplets would be. CX-1827C (Dear RWS) at Q/A 1142. "In short," Dr. Dear testifies, "this [redacted]ntry does not come close to showing conception of any claim in any 10X Asserted Patent." *Id.* at Q/A 1146-1147. *See* CX-1828C (Hindson RWS) at Q/A 16-19.

Specifically with respect to the draft provisional application, Dr. Dear notes that Dr. Heredia discusses [redacted] as disclosed in the 10X inventions. CX-1827C (Dear RWS) at Q/A 1156. Dr. Dear notes further that [redacted]

[redacted]. *Id.* at Q/A 1158. Dr. Heredia's [redacted] does not depict how [redacted] in other words, Dr. Heredia's idea "would not work without something not actually depicted." *Id.* at Q/A 1165. In sum, Dr. Dear demonstrates persuasively that Dr. Heredia's 2009 idea lacks the elements which, combined, interact to effectuate the patented invention.

Bio-Rad argues that an inventor need only contribute one individual feature to an invention. But the evidence, as discussed, does not support the contention that Dr. Heredia contributed even one element. Dr. Heredia himself does not point to anything his idea for a liquid bead contributed to the invention patented by 10X. *See, e.g.,* Ex. CX-0014C (Heredia Dep.) at 114:10-12 [redacted]

Bio-Rad lays great stress on case law that says each contributor need not have "their own contemporaneous picture" of the final claimed invention to qualify as a joint inventor. RIB at 13 (quoting *Vanderbilt,* 601 F.3d at 1302). Bio-Rad relies on case law holding that a contribution to individual features of a patented invention, "even at different times," may qualify for joint inventorship. *Id.* at 14. The evidence here does not establish clearly and convincingly that Dr. Heredia's work contributed to the patented technology at any time. Dr. Heredia did not conceive of anything at all that worked at the time he thought of it or that contributed to technology that was developed later. These facts distinguish this case from the cases Bio-Rad

cites in support of its arguments. "[O]ne who is 'too far removed from the real-world realization of an invention' is not a co-inventor." *Nartron,* 558 F.3d at 1359 (quoting *Eli Lilly & Co. v. Aradigm Corp.,* 376 F.3d 1352, 1359 (Fed. Cir. 2004)).

Bio-Rad argues that Dr. Heredia's disclosures share the "context" of the patented inventions. This is true only at a level of generality so high that it would render the concept of inventorship meaningless. As 10X asserts, "Dr. Heredia's [redacted] was a goal with no operative means to achieve it." CIB at 160. While Dr. Heredia's idea may have related generally to sample preparation and the "same sample preparation context," RRB at 61-62, he made no contribution toward meeting the goals of the invention in the way described in the patents. I cannot find clear and convincing evidence that Dr. Heredia's conception contributed at all, much less in a qualitative way, to the invention claimed in the asserted patents.

d. Insufficient evidence of significance

"'A joint inventor must contribute in some significant manner to the conception or reduction to practice of the invention [and] make a contribution to the claimed invention that is not insignificant in quality, when that contribution is measured against the dimension of the full invention.'" *Nartron,* 558 F.3d at 1356-57 (quoting *Pannu,* 155 F.3d at 1351).

Even Dr. Metzker admits that Dr. Heredia's idea "in isolation" is not a "significant contribution." *Id.* at Tr. 726:3-9. Dr. Heredia's idea, according to Dr. Metzker, is significant only if it [redacted] *Id.* at 728:14-22. In this respect, 10X and Bio-Rad seem to agree. *See* Tr. (Schnall-Levin) at 230:15-24 ("[T]his invention is not like a bag

of words, like barcodes, gel beads. It's actually how they're all put together, which is really important for driving the performance of the system."). Because there is no evidence that Dr. Heredia had an idea of how the elements that he allegedly conceived of would be put together to achieve the desired result, he made no significant contribution.

Absent evidence that Dr. Heredia's liquid bead contributed anything of significance to the patented technology (or any technology), Bio-Rad cannot demonstrate clearly and convincingly that Dr. Heredia is a joint inventor.

B. Ownership

As an affirmative defense to 10X's allegations of infringement, Bio-Rad claims ownership of each of the asserted patents in this investigation. 10X disputes Bio-Rad's claims of ownership, and Staff agrees with 10X. Although, in briefing the matter, the parties have lost their way in arguments concerning the law of inventorship, this is a contract dispute that boils down to a simple question: is there evidence that the idea embodied in the asserted patents was conceived by Drs. Hindson and Saxonov during the period in which they were employed by Quanta/Life and Bio-Rad? If the answer is yes, then as a matter of contract law, the asserted patents belong to Bio-Rad. If the answer is no, the asserted patents belong to 10X.

1. Legal Standards

"It is elementary that inventorship and ownership are separate issues." *Beech Aircraft Corp. v. EDO Corp.,* 990 F.3d 1237, 1248 (Fed. Cir. 1993). *Accord, Israel Bio-Eng'g Project v. Amgen, Inc.,* 475 F.3d 1256, 1263 (Fed. Cir. 2007) ("[I]ssues of patent ownership are distinct from questions of inventorship."). Ownership "is a question of who owns legal title to the subject matter in a patent," while "inventorship is a question of who actually invented the subject matter claimed in a patent." *Beech,* 990 F.2d at 1248. Bio-Rad confuses the issue by attempting

to use the legal analysis that applies to joint inventorship to resolve its ownership dispute with 10X. The distinction is illustrated in this case: the question whether Dr. Heredia should be treated as a co-inventor is one of inventorship; but there is no question that Drs. Hindson and Saxonov are inventors on the asserted patents. The question with respect to them is one of ownership, *i.e.,* do their contractual agreements with Bio-Rad and QuantaLife require that the asserted patents be assigned to Bio-Rad? *See FilmTec Corp. v. Hydranautics*, 982 F.2d 1546, 1550 (Fed. Cir. 1992) (stating that in a case that "turns on" "ownership", the court only needs "to decide whether the invention . . . was made or conceived" during the period of employment).[22]

Bio-Rad's ownership claims arise solely as the result of the contract terms governing the employment of Drs. Hindson and Saxonov, who are among the named inventors of the asserted patents. In general, contract terms must be construed under state law. *Board of Trustees of Leland Stanford Junior Univ. v. Roche Molecular Sys., Inc.,* 583 F.3d 832, 841 (citing *Jim Arnold Corp. v. Hydrotech Sys.,* 109 F.3d 1567, 1572 (Fed. Cir. 1997)). The exception to this

[22] Bio-Rad asserts that I adopted joint inventorship as a "guide" to ownership. RIB at 20 (citing Order No. 34). To the contrary, on reconsideration, Order. No. 41 clarified that "Order No. 34 did not conclusively establish the legal framework for deciding Bio-Rad's ownership claim." Order No. 41 at 2. In affirming denial of 10X's motion for summary determination on the ownership issue, Order No. 41 recognized that the "legal standard for addressing the ownership issue" continued to be disputed, and that "the parties' dispute would be better resolved after the conclusion of the evidentiary hearing, with the benefit of a complete evidentiary record regarding the contractual relationships between the parties and the contributions of the inventors." *Id.* As stated in Order No. 41, doubt concerning the facts and the law precluded a ruling on summary determination, including on the applicable legal standards. *See also Gen'l Elec. Co. v. Wilkins,* No. CV F 10-0674 LJO JLT, 2012 WL 3778865(E.D. Cal. 2012) at *19 note 3 ("[T]his Court is not bound by its interlocutory orders, which are not final, and may reconsider or modify them at any time.") (quoting *Marconi Wireless Telegraph Co. v. United States,* 320 U.S. 1, 63 (1943); *City of Los Angeles, Harbor Div. v. Santa Monica Baykeeper,* 254 F.3d 882(9th Cir. 2001)).

rule covers matters that are "intimately bound up with the question of standing in patent cases," such as "whether contractual language effects a present assignment of patent rights, or an agreement to assign rights in the future." *Id.* No such question is presented here.[23] This is important because the standard for determining joint ownership is a matter of patent law determined by federal courts, while the federal courts defer to state law on questions of contract. "[Q]uestions of contract law are matters of state law, questions related to patent law are interpreted according to federal law." *General Elec. Co. v. Wilkins,* No. CV F 10-0674 LJO JLT, 2012 WL 3778865 (E.D.Cal. 2012).

Confusing the two issues leads to error, as described by the federal court in *STMicroelectronics, Inc. v. Harari*, Case No. C 05-4691 JF, 2006 WL 2032580 (N.D. Cal. 2006).[24] In that case, the court addressed a dispute similar to the facts here: a company sued its former employee alleging that certain inventions were subject to a contract in which the employee agreed to assign inventions made during the term of his employment. *Id.* at *1-2. The district court initially found federal jurisdiction based on a substantial question of federal patent law. *Id.* at *2. The court reversed its decision on reconsideration, holding that "[o]wnership and inventorship issues are completely separate issues," and that the resolution of the ownership dispute depended entirely on the terms of the employment contract and the question of when "the

[23] Obviously, if Bio-Rad owns the patents, 10X lacks standing to assert them. But this is not a case in which there is a dispute concerning a present vs. a future assignment of rights, or how the actual assignment of patent rights among multiple parties affects standing. Interpretation of the contractual provisions, not application of the law of standing, determines the outcome in this instance.

[24] *Harari* is an unpublished decision. It is cited here not as precedent but as an instance in which a court mistakenly applied patent law inventorship principles to the issue of ownership, and thereafter recognized and corrected its mistake.

inventions described in the subject patents were 'made or conceived.'" *Id.* at *10-11. This "presen[ted] a factual question that does not implicate a substantial question of patent law," the court ruled, sending the case back to state court. *Id.* at *12-14.

The *Harari* court explicitly rejected the idea that determining the "inventive contribution" made by the employee mattered at all in deciding whether the company owned the inventions made by him. "It is unclear," the court lamented, "how Defendants, and subsequently the Court, came to inject the phrase 'inventive contribution' into the discussion of Harari's contractual disclosure and assignment obligations. The phrase does not appear in the Inventions Agreement . . . ." *Id.* at *8. Because the employment agreements required disclosure and assignment of all inventions and rights to inventions made during the term of employment, "there was no need to inquire into Harari's precise inventive contribution." *Id.* at *11.

Harari relies on *AT&T v. Integrated Network Corp.*, 972 F.2d 1321 (Fed. Cir. 1992), a precedential Federal Circuit decision involving similar facts, in which the Federal Circuit reversed a district court decision asserting jurisdiction and remanded with instructions to send the case to a state court. *Id.* at 1325. In *AT&T*, four employees left AT&T to join another firm, INC, "as a team." *Id.* at 1323. The employees were subject to agreements giving AT&T assignment rights in inventions made or conceived, either solely or jointly with others, during the course of their employment. *Id.*

The patent in question was filed about a year and a half later, naming the four former AT&T employees as inventors, and disclosing that the application for the patent was assigned to INC. *Id.* AT&T sued alleging that the invention in question had been disclosed in a proprietary [AT&T] memorandum prepared by one of the four employees during the period of employment. *Id.* AT&T alleged contract and tort claims. INC removed the case to federal district court, but

AT&T moved the district court to remand the case back to state court, arguing that it would seek to prove that the invention was conceived during the period of employment by AT&T, and that this did not present "a substantial question of federal patent law." *Id. Accord, e.g., ReCor Med. Inc. v. Warnking,* C.A. No. 7387-VCN, 2013 Del. Ch. LEXIS 142 at *34 (Del. Ch. Ct. May 31, 2013) ("[T]he Court can see no reason why patent law should displace contract law here." (citing *AT&T*).)

The district court kept the case but on appeal, the Federal Circuit held that it should be remanded to the state court for decision. *Id.* at 1324. The Circuit explained that "conception of inventions, as used in the employment agreement, is [not] solely a technical question of patent law." *Id.* Specifically, the Circuit opined that the moment "when an invention was conceived may be more a question of common sense than of patent law." *Id.* The Circuit said the state court was "free to look for guidance to the law on the conception of inventions as we may have explained it, but in light of the different facets of the word conceive, indeed of inventions, this may well not be determinative of the outcome . . . ." *Id.* at 1325 (quoting *Ingersoll-Rand Co. v. Ciavatta,* 542 A.2d 879 (1988). *Accord, e.g., Motorola, Inc. v. Lemko Corp.,* No. 08 C 5427, 2012 WL 74319, at *4-5 (N.D. Ill. Jan. 10, 2012) ("the parties did not necessarily use terms in their agreements in the same way in which they are defined in patent law").

While the jurisdictional question addressed in *AT&T* does not arise in a case brought pursuant to section 337, the principle is the same: where an action sounding in contract is brought, the resolution of the contract dispute should be decided based on state law, even in a patent case.[25] A state court may look to federal law for "guidance" on questions of inventorship

[25] *Ingersoll-Rand* states that it is the employer's burden to establish that conception occurred during the period of the employment contract. 542 A.2d at 894. *Accord, e.g., ReCor,* 2013 Del.

where that is appropriate, but an action for breach of contract remains a question of state law and does not arise under federal patent law. *AT&T, supra.* In the case before me, as in *AT&T*, the contract requires determination of when the idea that gave rise to the patents-in-issue was conceived. The time of conception, as the Circuit noted in *AT&T*, is not a patent law issue.

The parties in this case fall into the same trap bemoaned by the court in *Harari* to the extent that they argue about whether the concept of "complete inventorship" applies to Drs. Hindson and Saxonov. The notion of "complete inventorship" has no application with respect to ownership under the pertinent contracts. These contracts, like the contracts in *Harari*, are silent as to any inventive contribution, complete or incomplete, made by an employee. Under the unambiguous contract provisions, *see infra*, the only fact that matters is the actual time when the inventors conceived of the inventive idea embodied in the asserted patents. *See also Motorola,* 2012 WL 74319 at *5 ("[T]he terms 'developed or conceived . . . during the term of my employment' are not ambiguous. Their meaning is sufficiently clear that a jury could simply examine evidence of when the inventions or ideas embodied in the Lemko patents first came into existence in order to determine whether Pan and Labun's actions were within the scope of the contractual term.").

2. Discussion

The real dispute involves defining the inventive concept in the asserted patents. Bio-Rad has the burden to identify the idea of which it claims ownership. It has not done so. Instead, it has briefed the matter as if it owned a share of the patents because it could trace some elements of the asserted patents to work done at Quanta/Life and Bio-Rad. This is inconsistent with the

---

Ch. LEXIS 142 at *32 (employer "must show by a preponderance of the evidence that it is entitled to the relief it requested").

contracts: [redacted] [redacted] Bio-Rad, as 10X freely concedes, owns many ideas conceived by Drs. Hindson and Saxonov, but it does not own the idea for the specific arrangement of elements claimed in the asserted patents, as discussed herein, because there is insufficient evidence that that idea was conceived during the period of employment.

As described by Dr. Hindson, the invention claimed in the asserted patents is complex and consists of many elements. CX-0001C (Hindson WS) at Q/A 88. The inventive idea, which emerged from many other ideas (some of which clearly were in the prior art), is to combine these elements in a process resulting in what 10X calls the GEM ("gel bead in emulsion") architecture. As confirmed by both parties, the inventive idea is a specific arrangement of elements which, when combined, works to achieve a desired goal. *See* Tr. (Metzker) at 728:14-22 ("[I]t has to work within the architecture of a droplet, so partitioning the analyte from other analytes, having a reagent delivery system that adds the reagents that we can then combine, barcode, analyze and then track back to the different droplets, to what is the makeup of that analyte. All of that, all of that together is important."). *See also* Tr. (Schnall-Levin) at 230:15-24 ("[T]his invention is not like a bag of words, like barcodes, gel beads. It's actually how they're all put together, which is really important for driving the performance of the system."). The asserted patents each claim particular steps in the GEM architecture, and for purposes of ownership, the employment contracts at issue require determination of who conceived of this architecture and when. *See ReCor*, 2013 Del. Ch. Ct. LEXIS at *29, 42 (examining the record to determine when the "aha" or "eureka" moment occurred). Bio-Rad does not address squarely the critical contractual question of when the inventive concept in the asserted patents was conceived. Instead, Bio-Rad clouds the real issue with misplaced arguments about inventive contributions.

Consistent with the Federal Circuit's holding in *AT&T*, California law governs the pertinent employment agreements between Bio-Rad and Drs. Hindson and Saxonov. RX-0624C at ¶ 11; RX-0623C at ¶ 11; RX-0619C at ¶ 11; RX-0620C at ¶ 11. "Under California law, the interpretation of a written contract is a matter of law for the court even though questions of fact are involved." *Southland Corp. v. Emerald Oil Co.,* 789 F.2d 1441, 1443 (9th Cir.1986). Contract language that is plain and unambiguous requires no construction. "'In interpreting an unambiguous contractual provision we are bound to give effect to the plain and ordinary meaning of the language used by the parties.'" *Lockyer v. R.J. Reynolds Tobacco Co.,* 107 Cal. App. 4th 516, 517 (2003) (quoting *Coast Plaza Doctors Hospital v. Blue Cross of California,* 83 Cal. App. 4th 677, 684 (2000)). Where "'contract language is clear and explicit and does not lead to absurd results, we ascertain intent from the written terms and go no further.'" *Shaw v. Regents of Univ. of California,* 58 Cal. App. 4th 44, 53, 67 (1997). *See* Cal. Civ. Code § 1639 ("When a contract is reduced to writing, the intention of the parties is to be ascertained from the writing alone, if possible.").

The contracts in this case state, in pertinent part, with respect to QuantaLife:

[redacted]

And with respect to Bio-Rad:

[redacted]

RX-0619C at ¶¶ 3, 6; RX-0620C at ¶¶ 3, 6.

As set forth above, the QuantaLife contracts [redacted] RX-0623C at ¶2(a). The Bio-Rad contracts [redacted] RX-0619C at ¶¶3, 6. [redacted] *Id.* at ¶3.

No provision of any of the applicable contracts governs future inventions that are based on or developed from work done during employment. To the contrary, the plain, unambiguous contract language pertains only to ideas actually conceived during the employment period. Bio-

Rad's arguments improperly read out the plain meaning of the durational limitation in the pertinent contracts, and in its place suggest an interpretation of the contracts in which inventions developed by the employee after his employment belong to the company if they are related to ideas conceived during employment. "'When a dispute arises over the meaning of contract language, the first question to be decided is whether the language is 'reasonably susceptible' to the interpretation urged by the party. If it is not, the case is over.'" *Lockyer,* 107 Cal. App. 4th at 524. Bio-Rad's (implicit) construction is not reasonable.[26]

Bio-Rad's contention that "[b]ecause Hindson and Saxonov made contributions to the inventions that are now claimed in the Asserted Patents [redacted] . . . Bio-Rad has a pro rata undivided co-ownership interest in the Asserted Patents based on those contributions," RRB at 40, therefore is unavailing. Bio-Rad owns no interest in any of the patents unless it can demonstrate, in conformity with the contractual requirements, that Drs. Hindson and Saxonov actually conceived the inventive idea embodied in the asserted patents during the employment period. Bio-Rad does not cite to any provision of the employment contracts to support its contentions that an idea that is related to the invention embodied in the asserted patents, but is not the actual inventive idea in the asserted patents, confers ownership on Bio-Rad.

On review of this record, Bio-Rad has failed to present any direct evidence that the actual inventive idea embodied in the asserted patents was first conceived at Quanta/Life or Bio-Rad, as required by the contracts. Since it has presented no direct evidence of conception, Bio-Rad necessarily falls back on circumstantial evidence, asking me to infer that conception likely

[26] Bio-Rad has not actually offered any alternative construction of the contract terms.

occurred during the period of employment. Bio-Rad's argument is grounded mainly on the temporal proximity of Drs. Hindson and Saxonov's departure from Bio-Rad and the inventions developed thereafter by 10X.

These facts are basically undisputed: In October 2011, Bio-Rad acquired QuantaLife [redacted] RIB at 44 (citing RX-0502C (Tumolo DWS) at Q/A 32). Drs. Hindson and Saxonov worked at Bio-Rad for six months thereafter, leaving in what was a "coordinated event" in April 2012. *Id.* at 44-45 (citing Tr. (Hindson) at 162:3-9, 163:6-14; Tr. (Saxonov) at 797:4-21, 798:3-9). After taking off several months, Drs. Hindson and Saxonov formed 10X. Tr. (Hindson) at 163:3-164:3; CX-0001C (Hindson WS) at Q/A 38-40. Within four months of leaving Bio-Rad and less than a month after founding 10X, they filed their first provisional patent application at 10X on August 14, 2012, Provisional App. No. 61/683,192 (the "'192 application"). RX-0299.

This chronology alone does not establish circumstantially that the inventions at issue were conceived during Drs. Hindson and Saxonov's employment with QuantaLife and Bio-Rad. The circumstances of their departure make it likely that Drs. Hindson and Saxonov left Bio-Rad with the intention of pursuing opportunities to invent and market new technologies—they were free to do so. But these circumstances in themselves do not support a finding that Drs. Hindson and Saxonov conceived of the idea embodied in the asserted patents before they left Bio-Rad's employ.[27]

[27] The record indicates that Drs. Hindson and Saxonov left Bio-Rad because [redacted] Tr. (Saxonov) at 798:14-24. [redacted] *Id.* at 797:15-21.

Bio-Rad challenges Dr. Hindson's credibility, asking me to infer that he is lying about the time frame in which the inventive idea in the asserted patents was conceived. Bio-Rad maintains that the '192 provisional application, submitted in August 2012, refers to gel beads, and that that disclosure is inconsistent with Dr. Hindson's testimony that conception of the claimed porous gel beads did not occur until the [redacted]. *Id.* at 45 (citing CX-0001C (Hindson WS) at Q/A 85). In context, however, Dr. Hindson's testimony that [redacted] is not inconsistent with the '192 provisional. Dr. Hindson recalls [redacted] CX-0001C (Hindson WS) at Q/A 86. "Around that time or shortly thereafter, [redacted] *Id.* Bio-Rad has not pointed to any portion of the '192 provisional patent application showing that the idea to use porous gel beads to deliver barcodes was conceived [redacted] before the events described in detail by Dr. Hindson. [28]

Bio-Rad also points to a paper published in 2009 by inventors at Harvard, referred to as the "Beating Poisson" article. RIB at 47. The significance of the "Beating Poisson" article is that it discusses using microfluidics to deliver deformable gel beads to droplets that can be

[28] The '192 application states in pertinent part: "The microcapsules may also comprise a polymer within the interior of the capsule. In some instances this polymer may be a porous polymer bead that may entrap reagents or combinations of reagents. In other instances, this polymer may be a bead that has been previously swollen to create a gel." RX-0299 at ¶0050. This provision refers to a porous polymer bead that may entrap reagents but not to such a bead with barcodes or other reagents releasably attached, as in the asserted patents. As Staff notes, Bio-Rad's expert, Dr. Metzker, does not opine that the asserted claims were conceived in August 2012. SRB at 28 (citing Tr. (Metzker) at 705:2-22.)

functionalized with DNA. *Id.* *See* RX-0102. Dr. Hindson testifies in his direct witness statement he came across the "Beating Poisson" paper only in late 2012, while at 10X. CX-0001C (Hindson WS) at Q/A 132. On cross-examination, he concedes that he had encountered the paper in April 2011, while still at QuantaLife, but he claims not to have read it at that time. Tr. at 169:5-22, 172:8-18.

Bio-Rad maintains that Dr. Hindson's denial is implausible given the importance of the "Beating Poisson" article, pointing in particular to [redacted] RIB at 48; Tr. (Hindson) at 169:18-171:23). Bio-Rad maintains that Dr. Hindson's recollection also is undermined by [redacted] *Id.* (citing JX-0145C; Tr. (Saxonov) at 793:8-794:5.

I am not persuaded that this evidence undermines Dr. Hindson's credibility. I find it at least plausible that Dr. Hindson did not remember seeing the "Beating Poisson" article or [redacted]. The record does not indicate that Dr. Hindson attached particular significance to the article at that time, or that [redacted] indicated the conception of the idea for the inventions claimed in the asserted patents.[29] If Dr. Hindson did

[29] [redacted] JX-0145C. [redacted] does not indicate that Drs. Hindson and Saxonov at that time conceived the idea asserted in the patents. On the contrary, it indicates that they had not conceived the idea embodied in the patents at that time, because there is no mention of using porous gel beads or releasably attached oligonucleotides. The record shows that Drs. Hindson and Saxonov [redacted]

not realize in 2011 the significance of porous gel beads in the eventual development of the GEM architecture at 10X, it would be easy to forget [redacted] I conclude that Dr. Hindson's alleged lack of credibility is a slim reed for Bio-Rad to stand on.[30]

In addition to challenging Dr. Hindson's credibility, Bio-Rad points to evidence that certain concepts disclosed in Drs. Hindson and Saxonov's earlier work prefigured the patented invention. Presumably, Bio-Rad would contend (if Bio-Rad were attempting to establish conception under the correct legal theory) that because certain discoveries made by Drs. Hindson and Saxonov during the period of their employment included elements that also are found in the asserted patents, the particular arrangement of those elements, set forth in the asserted patents, must have occurred to them. For example, Bio-Rad discusses the concepts [redacted] RIB at 40-44. Dr. Saxonov testifies, however, that [redacted] CX-1829C

[redacted]. If that in itself were sufficient to trigger ownership of inventions patented after they left Bio-Rad, the contracts' [redacted] would be nullities.

[30] I agree with Bio-Rad that Dr. Hindson on several occasions was not forthcoming in his representations to Bio-Rad's representative about the work that was being conducted at 10X, but Dr. Hindson testifies credibly that he felt threatened by Bio-Rad; people are known to react defensively when they perceive they are under attack, even when they have done nothing wrong. *See* CX-1828C (Hindson RWS) at Q/A 55 ("It was very clear to me based on our conversations what she was asking me was 'are you using Quantalife droplets,' essentially fishing for whether we were competing with our old Quantalife products, and the answer to that was clearly 'no,' because we were using GEMs.")

(Saxonov RWS) at Q/A 22 ("We had not thought through these issues or come up with solutions that would have made it work.").

Bio-Rad also points out that certain number ranges of "cells, droplets, beads, and barcodes" were disclosed in the '059 patent, JX-0031, and that the numbers discussed in the claims of the asserted patents, as Dr. Saxonov concedes, could be derived easily based on those ranges. RRB at 55 (quoting RX-0412C (Saxonov Dep. Tr.) at 148:15-149:12). These facts do not demonstrate, even circumstantially, that the idea for the inventions claimed in the asserted patents had already been conceived at the time the '059 application was filed.[31]

Bio-Rad also contends that the entries in notebooks offered into evidence by 10X to support conception by the 10X inventors is "much more consistent with the theory that Dr. Hindson and others founded 10X to commercialize the ideas they had at QuantaLife and Bio-Rad." RRB at 57. Bio-Rad cites testimony from Dr. Schnall-Levin and Dr. Dear that allegedly corroborates Bio-Rad's argument that 10X's lab notebooks do not evidence the conception of the inventions claimed in the asserted patents. RIB at 130-131. Even assuming Bio-Rad's argument about the nature of 10X's notebooks is correct (and this is disputed), it would not necessarily

[31] 10X responds persuasively to each of the many circumstances alleged by Bio-Rad concerning the work done by Drs. Hindson and Saxonov during their period of employment, maintaining that their work was conducted in a variety of technological contexts distinct from the particular GEM architecture described in the asserted patents. *See* CIB at 139-151. It is not necessary or useful to try to resolve every one of the parties' disputes. These disputes are largely beside the point because, as discussed above, they are not probative on the issue of when the particular arrangement that constitutes the inventive concept of the asserted patents actually was conceived by Drs. Hindson and Saxonov.

lead to the conclusion that the claimed inventions were conceived at QuantaLife or Bio-Rad. Several months elapsed between the time Drs. Hindson and Saxonov left Bio-Rad and the founding of 10X. The actual idea could have been conceived at any time after Dr. Hindson and Saxonov left Bio-Rad's employ; the record does not indicate more likely than not that conception of the inventive idea in the asserted patents occurred before their departure.[32]

In sum, the evidence before me is insufficient to permit the conclusion that, more likely than not, the work Drs. Hindson and Saxonov did at QuantaLife and Bio-Rad led them to conceive the idea described in the 10X patents while they were still under contract. *Compare Agilent Techs., Inc. v. Kirkland,* C.A. No. 3512-VCS, 2010 WL 610725 at *15 (Del. Ch. Ct. Feb. 18, 2010) (finding employees conceived of technology at issue "based upon insights they formed and recorded at Agilent from observing the empirical results of experiments they conducted at Agilent"). Bio-Rad presents no pertinent records showing insights or experiments that support the argument that the inventive idea in the asserted patents was conceived before these employees left Bio-Rad. Given that Bio-Rad bears the burden of proof on this issue, Bio-Rad has failed to establish ownership of the asserted patents. *See* CX-1827C (Dear RWS) at Q/A 1129 ("Nothing [Dr. Metzker] cites shows whether there was a partial experiment involving some but not all of these elements. Nothing shows how the experiments would work. Nothing he cites shows any experimental observation. Dr. Metzker does not rely upon anything in his

[32] 10X's interrogatory responses detail with some specificity the timeline regarding development of the patented technology. *See* RX-0643C. In these responses, 10X seeks to show that the claims of the patents were conceived in [redacted]. *Id.* at 63-65. *See also* CX-1827C (Dear RWS) at Q/A 1269-1279. Dr. Metzker, Bio-Rad's witness, reviewed 10X's timeline regarding conception and declined to offer an opinion disagreeing with 10X's alleged [redacted] conception dates. Tr. (Metzker) at 704:7-705:22.

testimony offering a reasonable basis to conclude that any such experiments occurred nor, importantly, what happened in them.").

C. Other Affirmative Defenses

Bio-Rad raises several additional affirmative defenses. None has any merit.

Bio-Rad's equitable estoppel defense has two prongs. Bio-Rad contends that the employment agreements signed by Drs. Hindson and Saxonov [redacted] RIB at 132. First, the agreements cited by Bio-Rad give no such "explicit contractual assurances." Second, the evidence does not show that the inventions in the asserted 10X patents were made at QuantaLife and/or Bio-Rad.

Bio-Rad also contends that 10X is equitably estopped from bringing this action because Bio-Rad had no notice of infringement until this litigation was instituted, and Bio-Rad allegedly relied on 10X's "silence and inaction" in developing its product line "with the reasonable belief that it would not be subject to an infringement action." *Id.* at 133. Bio-Rad points to no evidence to support the contention that it relied on any lack of notice of infringement from 10X. *See id.* Bio-Rad's equitable estoppel defense fails for lack of proof.

Bio-Rad also claims to have an express license to practice each of the asserted patents, based on Drs. Hindson and Saxonov's [redacted] *Id.* at 133. Since Bio-Rad has not shown that the invention embodied in the 10X patents was made during the course of the employment agreement, this defense also fails.

Bio-Rad claims an implied license, wavier, and acquiescence based on "'circumstances [that] plainly indicate that the grant of a license should be inferred.'" *Id.* (quoting *Bandag, Inc. v. Al Bolser's Tire Stores, Inc.,* 750 F.2d 903, 925 (Fed. Cir. 1984)). This defense is based on the same contractual provisions asserted by Bio-Rad "with respect to Bio-Rad's affirmative defenses of acquiescence and equitable estoppel," and is rejected for the same reasons stated above. *See Id.* at 133. Bio-Rad has not demonstrated that Drs. Hindson and Saxonov were under any obligation to Bio-Rad with respect to the asserted 10X patents.

The "shop rights" defense similarly is predicated on the assertion that Drs. Hindson and Saxonov "'conceived' of the claimed inventions of the Asserted Patents while employed by and under contract at QuantaLife and/or Bio-Rad—or at the very least significantly and extensively contributed to the conception, development or making of the claimed inventions of the Asserted Patents – and did so using their employer's resources and personnel." *Id.* at 134-135.[33] As discussed above, the evidence does not show that Drs. Hindson and Saxonov conceived of the claimed inventions while under contract to QuantaLife and/or Bio-Rad, and the contracts that determine Bio-Rad's rights do not cover "contributions" made by employees during the course of their employment to inventions conceived after the employment ends. Accordingly, the shop rights doctrine affords Bio-Rad no defense.

[33] "The doctrine of shop rights has its origins in equity. A shop right is an employer's nonexclusive right to use an employee's patented process or invention that was developed during the employee's hours of employment. The right is based on the employer's presumed contribution to the invention through materials, time, and equipment." *California Eastern Labs., Inc. v. Gould,* 896 F.2d 400, 402 (9th Cir. 1990) (citing *U.S. v. Dubilier Condenser Corp.,* 239 U.S. 178 (1933)).

IX. CONCLUSIONS OF LAW

Based on the foregoing, and the record as a whole, it is my final initial determination that there is a violation of section 337 of the Tariff Act of 1930, as amended, 19 U.S.C. § 1337, in the importation into the United States, the sale for importation, and/or the sale within the United States after importation of certain microfluidic systems and components thereof and products containing same by reason of infringement of certain claims of U.S. Patent No. 9,689,024 ("the '024 Patent"), U.S. Patent No. 9,695,468 ("the '468 Patent"), and U.S. Patent No. 9,856,530 ("the '530 Patent"). There is no violation with respect to U.S. Patent No. 9,644,204 ("the '204 Patent").

This determination is based on the following conclusions of law:

1. The Commission has subject matter jurisdiction over this investigation, *in personam* jurisdiction over Bio-Rad, and *in rem* jurisdiction over the accused microfluidic systems and components thereof and products containing same.

2. There has been an importation into the United States, sale for importation, or sale within the United States after importation of the accused microfluidic systems and components thereof and products containing same by Bio-Rad.

3. Bio-Rad has indirectly infringed claims 1, 5, 17, 19, and 22 of the '024 patent with respect to its ddSEQ v1 products.

4. Bio-Rad has indirectly infringed claims 1, 6, 7, 9, and 21 of the '468 patent with respect to its ddSEQ v1 products.

5. 10X has not shown that any claims of the '204 patent are infringed by Bio-Rad.

6. Bio-Rad has indirectly infringed claims 1, 4, 11, 14, 19, 26, and 28 of the '530 patent with respect to its ddSEQ v1 products.

7. No claims of the '024 patent have been shown to be invalid.

8. No claims of the '468 patent have been shown to be invalid.

9. No claims of the '204 patent have been shown to be invalid.

10. No claims of the '530 patent have been shown to be invalid.

11. The domestic industry requirement is satisfied with respect to claims of the '024

patent.

12. The domestic industry requirement is satisfied with respect to claims of the '468 patent.

13. The domestic industry requirement is satisfied with respect to claims of the '204 patent.

14. The domestic industry requirement is satisfied with respect to claims of the '530 patent.

15. Bio-Rad has failed to carry its burden with respect to its allegations of improper inventorship, ownership, and other affirmative defenses.

I hereby certify the record in this investigation to the Commission with my final initial determination. Pursuant to Commission Rule 210.38, the record further comprises the Complaint and exhibits thereto filed with the Secretary, the *Markman* order, and the exhibits attached to the parties' summary determination motions and the responses thereto. 19 C.F.R. § 210.38(a).

Pursuant to Commission Rule 210.42(c), this initial determination shall become the determination of the Commission 45 days after the service thereof, unless a party files a petition for review pursuant to Commission Rule 210.43(a), the Commission orders its own review pursuant to Commission Rule 210.44, or the Commission changes the effective date of the initial determination. 19 C.F.R. § 210.42(h)(6).

This initial determination is being issued with a confidential designation pursuant to Commission Rule 210.5 and the protective order in this investigation. Within ten (10) days of the date of this initial determination, each party shall submit to the Administrative Law Judge a statement as to whether or not it seeks to have any portion of this document deleted from the public version. *See* 19 C.F.R. § 210.5(f). A party seeking to have a portion of this document deleted from the public version thereof must attach to its submission a copy of the document with red brackets indicating the portion(s) asserted to contain confidential business

information.[34] The parties' submissions under this subsection shall not be filed with the Commission Secretary but shall be submitted by paper copy to the Administrative Law Judge and by e-mail to the Administrative Law Judge's attorney advisor.

SO ORDERED.

Dee Lord
Administrative Law Judge

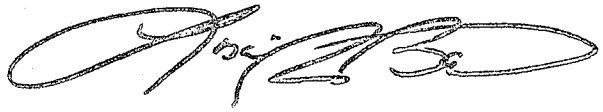
[34] To avoid depriving the public of the basis for understanding the result and reasoning underlying the decision, redactions should be limited. Parties who submit excessive redactions may be required to provide an additional written statement, supported by declarations from individuals with personal knowledge, justifying each proposed redaction and specifically explaining why the information sought to be redacted meets the definition for confidential business information set forth in Commission Rule 201.6(a). 19 C.F.R. § 201.6(a).

CERTAIN MICROFLUIDIC SYSTEMS AND COMPONENTS THEREOF AND PRODUCTS CONTAINING SAME

Inv. No. 337-TA-1100

PUBLIC CERTIFICATE OF SERVICE

I, Lisa R. Barton, hereby certify that the attached **INITIAL DETERMINATION** has been served by hand upon the Commission Investigative Attorney, **Monica Bhattacharyya, Esq.**, and the following parties as indicated, on **August 12, 2019.**



Lisa R. Barton, Secretary
U.S. International Trade Commission
500 E Street, SW, Room 112
Washington, DC 20436

On Behalf of Complainants 10X Genomics, Inc.:

Paul T. Ehrlich, Esq.
TENSEGRITY LAW GROUP LLP
555 Twin Dolphin Dr., Suite 650
Redwood Shores, CA 94061

- ☐ Via Hand Delivery
- ☑ Via Express Delivery
- ☐ Via First Class Mail
- ☐ Other:_____

On Behalf of Respondents Bio-Rad Laboratories, Inc.:

S. Alex Lasher, Esq.
QUINN EMANUEL URQUHART & SULLIVAN, LLP
1300 I Street NW, Suite 900
Washington, DC 20005

- ☐ Via Hand Delivery
- ☑ Via Express Delivery
- ☐ Via First Class Mail
- ☐ Other:_____

US009644204B2

(12) **United States Patent**
Hindson et al.

(10) **Patent No.: US 9,644,204 B2**
(45) **Date of Patent: May 9, 2017**

(54) **PARTITIONING AND PROCESSING OF ANALYTES AND OTHER SPECIES**

(71) Applicant: **10X Genomics, Inc.**, Pleasanton, CA (US)

(72) Inventors: **Benjamin Hindson**, Pleasanton, CA (US); **Serge Saxonov**, Oakland, CA (US); **Kevin Ness**, Pleasanton, CA (US); **Paul Hardenbol**, San Francisco, CA (US); **Christopher Hindson**, Pleasanton, CA (US); **Donald Masquelier**, Tracy, CA (US); **Mirna Jarosz**, Palo Alto, CA (US); **Michael Schnall-Levin**, Palo Alto, CA (US)

(73) Assignee: **10X GENOMICS, INC.**, Pleasanton, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 61 days.

(21) Appl. No.: **14/175,935**

(22) Filed: **Feb. 7, 2014**

(65) **Prior Publication Data**

US 2014/0227684 A1 Aug. 14, 2014

Related U.S. Application Data

(60) Provisional application No. 61/762,435, filed on Feb. 8, 2013, provisional application No. 61/800,223, filed on Mar. 15, 2013, provisional application No. 61/840,403, filed on Jun. 27, 2013, provisional application No. 61/844,804, filed on Jul. 10, 2013.

(51) **Int. Cl.**

C12Q 1/68	(2006.01)
C07H 21/00	(2006.01)
C12P 19/34	(2006.01)
G01N 33/50	(2006.01)
C12N 15/10	(2006.01)
C12N 15/11	(2006.01)
G01N 33/543	(2006.01)

(52) **U.S. Cl.**

CPC ***C12N 15/1065*** (2013.01); ***C12N 15/11*** (2013.01); ***C12Q 1/6806*** (2013.01); ***C12Q 1/6874*** (2013.01); ***C12Q 1/6876*** (2013.01); ***G01N 33/5436*** (2013.01); *C12Q 2600/112* (2013.01)

(58) **Field of Classification Search**

CPC C12Q 1/68; C12Q 1/6806; G01N 33/50

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,582,802	A	4/1986	Zimmerman et al.	
5,149,625	A	9/1992	Church et al.	
5,202,231	A	4/1993	Drmanac et al.	
5,413,924	A	5/1995	Kozak et al.	
5,436,130	A	7/1995	Mathies et al.	
5,512,131	A	4/1996	Kumar et al.	
5,587,128	A	12/1996	Wilding et al.	
5,605,793	A *	2/1997	Stemmer	C07K 14/43595 435/6.11
5,618,711	A	4/1997	Gelfand et al.	
5,695,940	A	12/1997	Drmanac et al.	
5,736,330	A	4/1998	Fulton	
5,756,334	A	5/1998	Perler et al.	
5,834,197	A	11/1998	Parton	
5,851,769	A	12/1998	Gray et al.	
5,856,174	A	1/1999	Lipshutz et al.	
5,958,703	A	9/1999	Dower et al.	
5,994,056	A	11/1999	Higuchi	
6,046,003	A	4/2000	Mandecki	
6,051,377	A	4/2000	Mandecki	
6,057,107	A	5/2000	Fulton	
6,103,537	A	8/2000	Ullman et al.	
6,143,496	A	11/2000	Brown et al.	
6,172,218	B1	1/2001	Brenner	
6,265,552	B1	7/2001	Schatz	
6,297,006	B1	10/2001	Drmanac et al.	
6,297,017	B1	10/2001	Schmidt et al.	
6,327,410	B1	12/2001	Walt et al.	
6,355,198	B1	3/2002	Kim et al.	
6,361,950	B1	3/2002	Mandecki	
6,372,813	B1	4/2002	Johnson et al.	
6,406,848	B1	6/2002	Bridgham et al.	
6,432,360	B1	8/2002	Church	
6,485,944	B1	11/2002	Church et al.	
6,511,803	B1	1/2003	Church et al.	
6,524,456	B1	2/2003	Ramsey et al.	
6,586,176	B1	7/2003	Trnovsky et al.	
6,632,606	B1	10/2003	Ullman et al.	

(Continued)

FOREIGN PATENT DOCUMENTS

EP	0249007 A2	12/1987
EP	0271281 A2	6/1988

(Continued)

OTHER PUBLICATIONS

Makino et al., Colloids and Surfaces B : Biointerfaces 12 :97 (1998).*

Nagashima et al.,Colloids and Surfaces B : Biointerfaces 12 :97 (1998).*

Office action dated Oct. 9, 2015 for U.S. Appl. No. 14/680,808.

U.S. Appl. No. 13/966,150, filed Aug. 13, 2013, Hindson et al.

U.S. Appl. No. 14/104,650, filed Dec. 12, 2013, Hindson et al.

U.S. Appl. No. 14/175,973, filed Feb. 7, 2014, Hindson et al.

Abate et al., Valve-based flow focusing for drog formation. Appl Phys Left. 2009;94. 3 pages.

(Continued)

Primary Examiner — Ethan C Whisenant

(74) *Attorney, Agent, or Firm* — Wilson Sonsini Goodrich & Rosati

(57) **ABSTRACT**

The present disclosure provides compositions, methods, systems, and devices for polynucleotide processing. Such polynucleotide processing may be useful for a variety of applications, including polynucleotide sequencing.

40 Claims, 16 Drawing Sheets

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,632,655	B1	10/2003	Mehta et al.	
6,670,133	B2	12/2003	Knapp et al.	
6,767,731	B2	7/2004	Hannah	
6,800,298	B1	10/2004	Burdick et al.	
6,806,052	B2	10/2004	Bridgham et al.	
6,806,058	B2	10/2004	Jesperson et al.	
6,859,570	B2	2/2005	Walt et al.	
6,884,788	B2	4/2005	Bulpitt et al.	
6,913,935	B1	7/2005	Thomas	
6,929,859	B2	8/2005	Chandler et al.	
6,969,488	B2	11/2005	Bridgham et al.	
6,974,669	B2	12/2005	Mirkin et al.	
7,041,481	B2	5/2006	Anderson et al.	
7,115,400	B1	10/2006	Adessi et al.	
7,129,091	B2	10/2006	Ismagilov et al.	
7,268,167	B2	9/2007	Higuchi et al.	
7,282,370	B2	10/2007	Bridgham et al.	
7,297,485	B2	11/2007	Bornarth et al.	
7,323,305	B2	1/2008	Leamon et al.	
7,425,431	B2	9/2008	Church et al.	
7,536,928	B2	5/2009	Kazuno	
7,604,938	B2	10/2009	Takahashi et al.	
7,622,280	B2	11/2009	Holliger et al.	
7,638,276	B2	12/2009	Griffiths et al.	
7,645,596	B2	1/2010	Williams et al.	
7,666,664	B2	2/2010	Sarofim et al.	
7,708,949	B2	5/2010	Stone et al.	
7,709,197	B2	5/2010	Drmanac	
7,745,178	B2	6/2010	Dong	
7,776,927	B2	8/2010	Chu et al.	
RE41,780	E	9/2010	Anderson et al.	
7,799,553	B2	9/2010	Mathies et al.	
7,842,457	B2	11/2010	Berka et al.	
7,901,891	B2	3/2011	Drmanac et al.	
7,910,354	B2	3/2011	Drmanac et al.	
7,960,104	B2	6/2011	Drmanac et al.	
7,968,287	B2	6/2011	Griffiths et al.	
7,972,778	B2	7/2011	Brown et al.	
8,003,312	B2	8/2011	Krutzik et al.	
8,053,192	B2	11/2011	Bignell et al.	
8,067,159	B2	11/2011	Brown et al.	
8,133,719	B2	3/2012	Drmanac et al.	
8,252,539	B2	8/2012	Quake et al.	
8,268,564	B2	9/2012	Roth et al.	
8,273,573	B2	9/2012	Ismagilov et al.	
8,278,071	B2	10/2012	Brown et al.	
8,304,193	B2	11/2012	Ismagilov et al.	
8,329,407	B2	12/2012	Ismagilov et al.	
8,337,778	B2	12/2012	Stone et al.	
8,592,150	B2	11/2013	Drmanac et al.	
8,603,749	B2	12/2013	Gillevet	
8,679,756	B1	3/2014	Brenner et al.	
8,748,094	B2	6/2014	Weitz et al.	
8,748,102	B2	6/2014	Berka et al.	
8,765,380	B2	7/2014	Berka et al.	
8,822,148	B2	9/2014	Ismagliov et al.	
8,835,358	B2	9/2014	Fodor et al.	
8,871,444	B2	10/2014	Griffiths et al.	
8,889,083	B2	11/2014	Ismagilov et al.	
8,986,286	B2	3/2015	Tanghoj et al.	
9,012,390	B2	4/2015	Holtze et al.	
9,017,948	B2	4/2015	Agresti et al.	
9,029,083	B2	5/2015	Griffiths et al.	
9,249,460	B2	2/2016	Pushkarev et al.	
9,347,059	B2	5/2016	Saxonov	
9,388,465	B2	7/2016	Hindson et al.	
9,410,201	B2	8/2016	Hindson et al.	
2001/0020588	A1	9/2001	Adourian et al.	
2001/0044109	A1	11/2001	Mandecki	
2002/0034737	A1	3/2002	Drmanac	
2002/0051992	A1	5/2002	Bridgham et al.	
2002/0089100	A1	7/2002	Kawasaki	
2002/0092767	A1	7/2002	Bjornson et al.	
2002/0179849	A1	12/2002	Maher et al.	
2003/0008285	A1	1/2003	Fischer	
2003/0008323	A1	1/2003	Ravkin et al.	
2003/0027221	A1*	2/2003	Scott	C12Q 1/68 435/7.21
2003/0028981	A1	2/2003	Chandler et al.	
2003/0039978	A1	2/2003	Hannah	
2003/0044777	A1	3/2003	Beattie	
2003/0044836	A1	3/2003	Levine et al.	
2003/0082587	A1	5/2003	Seul et al.	
2003/0104466	A1	6/2003	Knapp et al.	
2003/0108897	A1	6/2003	Drmanac	
2003/0149307	A1	8/2003	Hai et al.	
2003/0170698	A1	9/2003	Gascoyne et al.	
2003/0182068	A1	9/2003	Battersby et al.	
2003/0207260	A1*	11/2003	Trnovsky	C12Q 1/68 435/5
2003/0215862	A1	11/2003	Parce et al.	
2004/0063138	A1	4/2004	McGinnis et al.	
2004/0132122	A1	7/2004	Banerjee et al.	
2004/0258701	A1*	12/2004	Dominowski	A61K 9/1075 424/184.1
2005/0019839	A1	1/2005	Jesperson et al.	
2005/0042625	A1	2/2005	Schmidt et al.	
2005/0079510	A1*	4/2005	Berka	B01L 3/502707 506/16
2005/0130188	A1	6/2005	Walt et al.	
2005/0172476	A1	8/2005	Stone et al.	
2005/0181379	A1	8/2005	Su et al.	
2005/0202429	A1*	9/2005	Trau	B01J 13/02 435/6.16
2005/0202489	A1	9/2005	Cho et al.	
2005/0221339	A1	10/2005	Griffiths et al.	
2005/0244850	A1	11/2005	Huang et al.	
2005/0287572	A1	12/2005	Mathies et al.	
2006/0002890	A1	1/2006	Hersel et al.	
2006/0020371	A1	1/2006	Ham et al.	
2006/0073487	A1	4/2006	Oliver et al.	
2006/0078888	A1	4/2006	Griffiths et al.	
2006/0153924	A1	7/2006	Griffiths et al.	
2006/0163385	A1	7/2006	Link et al.	
2006/0199193	A1	9/2006	Koo et al.	
2006/0240506	A1	10/2006	Kushmaro et al.	
2006/0257893	A1	11/2006	Takahashi et al.	
2006/0263888	A1	11/2006	Fritz et al.	
2006/0292583	A1	12/2006	Schneider et al.	
2007/0003442	A1	1/2007	Link et al.	
2007/0020617	A1	1/2007	Trnovsky et al.	
2007/0054119	A1	3/2007	Garstecki et al.	
2007/0077572	A1	4/2007	Tawfik et al.	
2007/0092914	A1	4/2007	Griffiths et al.	
2007/0099208	A1	5/2007	Drmanac et al.	
2007/0154903	A1	7/2007	Marla et al.	
2007/0160503	A1	7/2007	Sethu et al.	
2007/0172873	A1	7/2007	Brenner et al.	
2007/0190543	A1*	8/2007	Livak	C12Q 1/6816 435/6.19
2007/0195127	A1	8/2007	Ahn et al.	
2007/0207060	A1	9/2007	Zou et al.	
2007/0228588	A1	10/2007	Noritomi et al.	
2007/0264320	A1	11/2007	Lee et al.	
2008/0003142	A1	1/2008	Link et al.	
2008/0004436	A1	1/2008	Tawfik et al.	
2008/0014589	A1	1/2008	Link et al.	
2008/0213766	A1	9/2008	Brown et al.	
2008/0228268	A1	9/2008	Shannon et al.	
2008/0241820	A1	10/2008	Krutzik et al.	
2009/0005252	A1	1/2009	Drmanac et al.	
2009/0011943	A1	1/2009	Drmanac et al.	
2009/0012187	A1*	1/2009	Chu	A61K 9/113 516/54
2009/0025277	A1*	1/2009	Takanashi	C10G 31/08 44/302
2009/0035770	A1	2/2009	Mathies et al.	
2009/0048124	A1	2/2009	Leamon et al.	
2009/0053169	A1	2/2009	Castillo et al.	
2009/0068170	A1	3/2009	Weitz et al.	
2009/0098555	A1	4/2009	Roth et al.	
2009/0118488	A1	5/2009	Drmanac et al.	
2009/0137404	A1	5/2009	Drmanac et al.	

(56) **References Cited**

U.S. PATENT DOCUMENTS

2009/0137414 A1 5/2009 Drmanac et al.
2009/0143244 A1 6/2009 Bridgham et al.
2009/0148961 A1 6/2009 Luchini et al.
2009/0155781 A1 6/2009 Drmanac et al.
2009/0197248 A1 8/2009 Griffiths et al.
2009/0197772 A1 8/2009 Griffiths et al.
2009/0202984 A1 8/2009 Cantor
2009/0203531 A1 8/2009 Kurn
2009/0264299 A1 10/2009 Drmanac et al.
2009/0286687 A1 11/2009 Dressman et al.
2010/0021973 A1 1/2010 Makarov et al.
2010/0021984 A1 1/2010 Edd et al.
2010/0022414 A1 1/2010 Link et al.
2010/0035254 A1 2/2010 Williams
2010/0062494 A1 3/2010 Church et al.
2010/0069263 A1 3/2010 Shendure et al.
2010/0105112 A1 4/2010 Holtze et al.
2010/0130369 A1 5/2010 Shenderov et al.
2010/0136544 A1 6/2010 Agresti et al.
2010/0137163 A1 6/2010 Link et al.
2010/0173394 A1 7/2010 Colston, Jr. et al.
2010/0210479 A1 8/2010 Griffiths et al.
2011/0033548 A1 2/2011 Lai et al.
2011/0033854 A1 2/2011 Drmanac et al.
2011/0053798 A1 3/2011 Hindson et al.
2011/0071053 A1 3/2011 Drmanac et al.
2011/0086780 A1 4/2011 Colston, Jr. et al.
2011/0092376 A1 4/2011 Colston, Jr. et al.
2011/0092392 A1 4/2011 Colston, Jr. et al.
2011/0160078 A1 6/2011 Fodor et al.
2011/0195496 A1 8/2011 Muraguchi et al.
2011/0201526 A1 8/2011 Berka et al.
2011/0217736 A1 9/2011 Hindson
2011/0218123 A1 9/2011 Weitz et al.
2011/0263457 A1 10/2011 Krutzik et al.
2011/0267457 A1 11/2011 Weitz et al.
2011/0281738 A1 11/2011 Drmanac et al.
2011/0305761 A1 12/2011 Shum et al.
2011/0319281 A1 12/2011 Drmanac
2012/0000777 A1 1/2012 Garrell et al.
2012/0010098 A1 1/2012 Griffiths et al.
2012/0010107 A1 1/2012 Griffiths et al.
2012/0015382 A1 1/2012 Weitz et al.
2012/0015822 A1 1/2012 Weitz et al.
2012/0071331 A1 3/2012 Casbon et al.
2012/0121481 A1 5/2012 Romanowsky et al.
2012/0132288 A1 5/2012 Weitz et al.
2012/0135893 A1 5/2012 Drmanac et al.
2012/0172259 A1 7/2012 Rigatti et al.
2012/0190032 A1 7/2012 Ness et al.
2012/0196288 A1 8/2012 Beer
2012/0211084 A1 8/2012 Weitz et al.
2012/0220494 A1 8/2012 Samuels et al.
2012/0220497 A1 8/2012 Jacobson et al.
2012/0222748 A1 9/2012 Weitz et al.
2012/0309002 A1 12/2012 Link
2012/0316074 A1 12/2012 Saxonov
2013/0018970 A1 1/2013 Woundy et al.
2013/0028812 A1 1/2013 Prieto et al.
2013/0046030 A1 2/2013 Rotem et al.
2013/0078638 A1 3/2013 Berka et al.
2013/0079231 A1 3/2013 Pushkarev et al.
2013/0109575 A1 5/2013 Kleinschmidt et al.
2013/0157870 A1 6/2013 Pushkarev et al.
2013/0157899 A1 6/2013 Adler, Jr. et al.
2013/0178368 A1 7/2013 Griffiths et al.
2013/0189700 A1* 7/2013 So G01N 1/34 435/6.12
2013/0203675 A1 8/2013 Desimone et al.
2013/0210639 A1 8/2013 Link et al.
2013/0210991 A1 8/2013 Fonnum et al.
2013/0211055 A1 8/2013 Raines et al.
2013/0225418 A1 8/2013 Watson
2013/0274117 A1 10/2013 Church et al.
2013/0296173 A1 11/2013 Callow et al.
2013/0343317 A1 12/2013 Etemad et al.
2014/0037514 A1 2/2014 Stone et al.
2014/0057799 A1 2/2014 Johnson et al.
2014/0065234 A1 3/2014 Shum et al.
2014/0155295 A1 6/2014 Hindson et al.
2014/0194323 A1 7/2014 Gillevet
2014/0199730 A1 7/2014 Agresti et al.
2014/0199731 A1 7/2014 Agresti et al.
2014/0206554 A1 7/2014 Hindson et al.
2014/0227706 A1* 8/2014 Kato C12Q 1/6886 435/6.12
2014/0228255 A1 8/2014 Hindson et al.
2014/0235506 A1 8/2014 Hindson et al.
2014/0287963 A1 9/2014 Hindson et al.
2014/0302503 A1 10/2014 Lowe et al.
2014/0378322 A1 12/2014 Hindson et al.
2014/0378345 A1 12/2014 Hindson et al.
2014/0378349 A1 12/2014 Hindson et al.
2014/0378350 A1 12/2014 Hindson et al.
2015/0005199 A1 1/2015 Hindson et al.
2015/0005200 A1 1/2015 Hindson et al.
2015/0011430 A1 1/2015 Saxonov
2015/0011432 A1 1/2015 Saxonov
2015/0111256 A1* 4/2015 Church C12Q 1/6851 435/91.2
2015/0119280 A1 4/2015 Srinivas et al.
2015/0218633 A1 8/2015 Hindson et al.
2015/0224466 A1 8/2015 Hindson et al.
2015/0225777 A1 8/2015 Hindson et al.
2015/0225778 A1 8/2015 Hindson et al.
2015/0292988 A1 10/2015 Bharadwaj et al.
2015/0298091 A1 10/2015 Weitz et al.
2015/0299772 A1 10/2015 Zhang
2015/0376605 A1 12/2015 Jarosz et al.
2015/0376609 A1 12/2015 Hindson et al.
2015/0376700 A1 12/2015 Schnall-Levin et al.
2015/0379196 A1 12/2015 Schnall-Levin et al.
2016/0024558 A1 1/2016 Hardenbol et al.
2016/0122817 A1 5/2016 Jarosz et al.
2016/0244742 A1 8/2016 Linnarsson et al.
2016/0257984 A1 9/2016 Hardenbol et al.
2016/0304860 A1 10/2016 Hindson et al.

FOREIGN PATENT DOCUMENTS

EP 0637996 B1 7/1997
EP 1019496 B1 9/2004
EP 1482036 B1 10/2007
EP 1594980 B1 11/2009
EP 1967592 B1 4/2010
EP 2258846 A2 12/2010
EP 2145955 B1 2/2012
EP 1905828 B1 8/2012
EP 2136786 B1 10/2012
EP 1908832 B1 12/2012
EP 2540389 A1 1/2013
EP 2752664 A1 7/2014
GB 2485850 A 5/2012
JP S 5949832 A 3/1984
JP 2006-507921 A 3/2006
JP 2006-289250 A 10/2006
JP 2007-268350 A 10/2007
JP 2009-208074 A 9/2009
WO WO-9530782 A1 11/1995
WO WO 96/29629 A2 3/1996
WO WO 96/41011 A1 12/1996
WO WO 99/09217 A1 2/1999
WO WO 99/52708 A1 10/1999
WO WO 00/08212 A1 2/2000
WO WO 00/26412 A1 5/2000
WO WO-0070095 A2 11/2000
WO WO 01/14589 A2 3/2001
WO WO 01/89787 A2 11/2001
WO WO 02/31203 A2 4/2002
WO WO 02/086148 A1 10/2002
WO WO 2004/002627 A2 1/2004
WO WO 2004/010106 A2 1/2004
WO WO-2004069849 A2 8/2004
WO WO 2004/091763 A2 10/2004

(56) **References Cited**

FOREIGN PATENT DOCUMENTS

WO	WO 2004/102204	A1	11/2004
WO	WO 2004/103565	A2	12/2004
WO	WO 2004/105734	A1	12/2004
WO	WO-2005002730	A1	1/2005
WO	WO 2005/082098	A2	2/2005
WO	WO 2005/021151	A1	3/2005
WO	WO 2005/023331	A2	3/2005
WO	WO 2005/040406	A1	5/2005
WO	WO 2005/049787	A2	6/2005
WO	WO 2006/030993	A1	3/2006
WO	WO 2006/078841	A1	7/2006
WO	WO 2006/096571	A2	9/2006
WO	WO 2007/001448	A2	1/2007
WO	WO 2007/002490	A2	1/2007
WO	WO 2007/024840	A2	3/2007
WO	WO 2007/081385	A2	7/2007
WO	WO 2007/081387	A1	7/2007
WO	WO 2007/089541	A2	8/2007
WO	WO 2007/114794	A1	10/2007
WO	WO 2007/121489	A2	10/2007
WO	WO 2007/133710	A2	11/2007
WO	WO 2007/138178	A2	12/2007
WO	WO 2007/139766	A2	12/2007
WO	WO 2007/140015	A2	12/2007
WO	WO 2007/149432	A2	12/2007
WO	WO 2008/091792	A2	1/2008
WO	WO 2008/021123	A1	2/2008
WO	WO 2008/102057	A1	8/2008
WO	WO 2008/109176	A2	9/2008
WO	WO 2008/121342	A2	10/2008
WO	WO 2008/134153	A1	11/2008
WO	WO 2009/005680	A1	1/2009
WO	WO 2009/011808	A1	1/2009
WO	WO 2009/061372	A1	5/2009
WO	WO 2009/085215	A1	7/2009
WO	WO 2010/004018	A2	1/2010
WO	WO 2010/033200	A2	3/2010
WO	WO-2010115154	A1	10/2010
WO	WO 2010/148039	A2	12/2010
WO	WO 2010/151776	A2	12/2010
WO	WO 2011/047870	A1	4/2011
WO	WO 2011/056546	A1	5/2011
WO	WO 2011/066476	A1	6/2011
WO	WO-2011074960	A1	6/2011
WO	WO 2012/012037	A1	1/2012
WO	WO 2012/048341	A1	4/2012
WO	WO 2012/083225	A2	6/2012
WO	WO-2012106546	A2	8/2012
WO	WO-2012112970	A2	8/2012
WO	WO-2012142611	A2	10/2012
WO	WO 2012/149042	A2	11/2012
WO	WO 2012/166425	A2	12/2012
WO	WO-2013019751	A1	2/2013
WO	WO-2013036929	A1	3/2013
WO	WO-2013122996	A1	8/2013
WO	WO-2013123125	A1	8/2013
WO	WO 2013/177220	A1	11/2013
WO	WO 2014/028537	A1	2/2014
WO	WO-2014074611	A1	5/2014
WO	WO-2014093676	A1	6/2014
WO	WO-2014210353	A2	12/2014
WO	WO-2015044428	A1	4/2015
WO	WO-2015164212	A1	10/2015

OTHER PUBLICATIONS

Abate, et al. High-throughput injection with microfluidics using picoinjectors. Proc Natl Acad Sci U S A. Nov. 9, 2010;107(45):19163-6. doi: 10.1073/pNas.1006888107. Epub Oct. 20, 2010.

Advisory Action mailed Nov. 20, 2013 for U.S. Appl. No. 13/139,326.

Agresti, et al. Selection of ribozymes that catalyse multiple-turnover Diels-Alder cycloadditions by using in vitro compartmentalization. Proc Natl Acad Sci U S A. Nov. 8, 2005;102(45):16170-5. Epub Oct. 31, 2005.

Akselband, "Enrichment of slow-growing marine microorganisms from mixed cultures using gel microdrop (GMD) growth assay and fluorescence-activated cell sorting", J. Exp. Marine Bioi., 329: 196-205 (2006).

Akselband, "Rapid mycobacteria drug susceptibility testing using gel microdrop (GMD) growth assay and flow cytometry", J. Microbiol. Methods, 62:181-197 (2005).

Anna et al., "Formation of dispersions using 'flow focusing' in microchannels", Appln. Phys. Letts. 82:3 364 (2003).

Braeckmans et al., Scanning the Code. Modern Drug Discovery. 2003:28-32.

Bransky, et al. A microfluidic droplet generator based on a piezo-electric actuator. Lab Chip. Feb. 21, 2009;9(4):516-20. doi: 10.1039/b814810d. Epub Nov. 20, 2008.

Carroll, "The selection of high-producing cell lines using flow cytometry and cell sorting", Exp. Op. Bioi. Therp., 4:11 1821-1829 (2004).

Chaudhary "A rapid method of cloning functioNal variable-region antibody genese in *Escherichia coli* as single-chain immunotoxins" Proc. Nat!. Acad. Sci USA 87: 1066-1070 (Feb. 1990).

Chechetkin et al., Sequencing by hybridization with the generic 6-mer oligonucleotide microarray: an advanced scheme for data processing. J Biomol Struct Dyn. Aug. 2000;18(1):83-101.

Chinese Office Action and search report mailed May 23, 2013 for Application No. CN 200880127116.4.

Chinese office action dated Jun. 18, 2012 for CN Application No. 200880127116.4.

Clausell-Tormos et al., "Droplet-based microfluidic platforms for the encapsulation and screening of mammalian cells and multicellular organisms", Chem. Biol. 15:427-437 (2008).

De Bruin et al., UBS Investment Research. Q-Series®: DNa Sequencing. UBS Securities LLC. Jul. 12, 2007. 15 pages.

Demirci, et al. Single cell epitaxy by acoustic picolitre droplets. Lab Chip. Sep. 2007;7(9):1139-45. Epub Jul. 10, 2007.

Doerr, "The smallest bioreactor", Nature Methods, 2:5 326 (2005).

Dowding, et al. Oil core/polymer shell microcapsules by interNal phase separation from emulsion droplets. II: controlling the release profile of active molecules. Langmuir. Jun. 7, 2005;21(12):5278-84.

Drmanac et al., Sequencing by hybridization (SBH): advantages, achievements, and opportunities. Adv Biochem Eng Biotechnol. 2002;77 :75-101.

Droplet Based Sequencing (slides) dated (Mar. 12, 2008).

Esser-Kahn, et al. Triggered release from polymer capsules. Macromolecules. 2011; 44:5539-5553.

European office action dated Jan. 23, 2012 for Application No. EP 08865992.5.

European office action dated Apr. 5, 2013 for Application No. EP 08865992.5.

European office action dated Aug. 29, 2013 for Application No. EP 08865992.5.

European office action dated Dec. 15, 2010 for EP Application No. 08865992.5.

Freiberg, et al. Polymer microspheres for controlled drug release. Int J Pharm. Sep. 10, 2004;282(1-2):1-18.

Fu, "A micro fabricated fluorescence-activated cell sorter", Nature Biotech., 17:1109-1111 (1997).

Fulton et al., Advanced multiplexed analysis with the FlowMetrix system. Clin Chern. Sep. 1997;43(9): 1749-56.

Garstecki, et al. Formation of monodisperse bubbles in a microfluidic flow-focusing device. Applied Physics Letters. 2004; 85(13):2649-2651. DOI: 10.1063/1.1796526.

Hashimshony, et al. CEL-Seq: Single-Cell RNa-Seq by Multiplexed Linear Amplification. Cell Rep. Sep. 27, 2012;2(3):666-73. doi: 10.1016/j.celrep.2012.08.003. Epub Aug. 30, 2012.

He "Selective Encapsulation of Single Cells and Subcellular Organelles into Picoliter- and Femtoliter-Volume Droplets" ANal. Chern 77: 1539-1544 (2005).

Holtze, et al. Biocompatible surfactants for water-in-fluorocarbon emulsions. Lab Chip. Oct. 2008;8(10):1632-9. doi: 10.1039/b806706f. Epub Sep. 2, 2008.

(56) **References Cited**

OTHER PUBLICATIONS

Huebner, "Quantitative detection of protein expression in single cells using droplet microfluidics", Chern. Commun 1218-1220 (2007).
International Preliminary Report on Patentability dated Mar. 31, 2011 for PCT/US09/005184.
International Preliminary Report on Patentability dated May 10, 2012 for PCT/US2010/054050.
International Preliminary Report on Patentability dated Jun. 30, 2011 for PCT/US2009/006649.
International Preliminary Report on Patentability dated Jul. 1, 2010 for PCT/US2008/013912.
International Preliminary Report on Patentability dated Sep. 17, 2009 for PCT/US2008/003185 mailed Sep. 17, 2009.
International search report and written opinion dated Jan. 12, 2009 for PCT/US2008/003185.
International search report and written opinion dated Jan. 31, 2011 for PCT/US2010/054050.
International search report and written opinion dated Apr. 3, 2009 for PCT/US2008/013912.
International search report and written opinion dated Aug. 16, 2010 for PCT/US2009/005184.
International search report and written opinion dated Oct. 2, 2009 for PCT/US2009/004037.
International search report and written opinion dated Oct. 21, 2009 for PCT/US2009/003389.
International search report and written opinion dated Oct. 29, 2008 for PCT/US2008/008563.
International search report and written opinion dated Dec. 16, 2013 for PCT/US2013/054797.
International search report dated Apr. 22, 2009 for PCT/US2009/000664.
Japanese Office Action and mailed Jul. 17, 2013 for Application No. JP 2010-539498.
Japanese Office Action mailed Nov. 19, 2013 for Application No. JP 2012-536941.
Khomiakov A et al., [Analysis of perfect and mismatched DNA duplexes by a generic hexanucleotide microchip]. Mol Bioi (Mosk). Jul.-Aug. 2003;37(4):726-41. Russian. Abstract only.
Kim, et al. Albumin loaded microsphere of amphiphilic poly(ethylene glycol)/poly(alpha-ester) multiblock copolymer. Eur J Pharm Sci. Nov. 2004;23(3):245-51.
Kim, et al. Fabrication of monodisperse gel shells and functioNal microgels in microfluidic devices. Angew Chem Int Ed Engl. 2007;46(11):1819-22.
Koster et al., "Drop-based microfluidic devices for encapsulation of single cells", Lab on a Chip the Royal Soc. of Chern. 8: 1110-1115 (2008).
Kutyavin, et al. Oligonucleotides containing 2-aminoadenine and 2-thiothymine act as selectively binding complementary agents. Biochemistry. Aug. 27, 1996;35(34):11170-6.
Li, Y., et al., "PEGylated PLGA Nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats," JourNal of Controlled Release, vol. 71, pp. 203-211 (2001).
Liu, et al. Preparation of uniform-sized PLA microcapsules by combining Shirasu porous glass membrane emulsification technique and multiple emulsion-solvent evaporation method. J Control Release. Mar. 2, 2005;103(1):31-43. Epub Dec. 21, 2004.
Liu, et al. Smart thermo-triggered squirting capsules for Nanoparticle delivery. Soft Matter. 2010; 6(16):3759-3763.
Loscertales, I.G., et al., "Micro/Nano Encapsulation via Electrified Coaxial Liquid Jets," Science, vol. 295, pp. 1695-1698 (2002).
Love, "A microengraving method for rapid selection of single cells producing antigen-specific antibodies", Nature Biotech, 24:6 703 (Jun. 2006).
Marcus. Gene method offers diagnostic hope. The Wall Street JourNal. Jul. 11, 2012.
Merriman, et al. Progress in ion torrent semiconductor chip based sequencing. Electrophoresis. Dec. 2012;33(23):3397-417. doi: 10.1002/elps.201200424.
Mirzabekov, "DNA Sequencing by Hybridization—a Megasequencing Method and a Diagnostic Tool?" Trends in Biotechnology 12(1): 27-32 (1994).
Mouritzen et al., Single nucleotide polymorphism genotyping using locked nucleic acid (LNa). Expert Rev Mol Diagn. Jan. 2003;3(1):27-38.
Nguyen, et al. In situ hybridization to chromosomes stabilized in gel microdrops. Cytometry. 1995; 21:111-119.
Oberholzer, et al. Polymerase chain reaction in liposomes. Chem Biol. Oct. 1995;2(10):677-82.
Office action dated Jan. 4, 2010 for U.S. Appl. No. 12/172,186.
Office action dated Feb. 28, 2013 for U.S. Appl. No. 13/139,326.
Office action dated Apr. 24, 2013 for U.S. Appl. No. 13/119,470.
Office action dated Aug. 6, 2013 for U.S. Appl. No. 13/139,326.
Office action dated Sep. 17, 2013 for U.S. Appl. No. 13/503,588.
Ogawa, et al. Production and characterization of O/W emulsions containing cationic droplets stabilized by lecithin-chitosan membranes. J Agric Food Chem. Apr. 23, 2003;51(9):2806-12.
Okushima, "Controlled production of monodisperse double emulsions by two-step droplet breakup in microfluidic devices", Langmuir, 20:9905-9908 (2004).
Perez, C., et al., "Poly(lactic acid)-poly(ethylene glycol) Nanoparticles as new carriers for the delivery ofplasmid DNa," JourNal of Controlled Release, vol. 75, pp. 211-224 (2001).
Peters, et al. Accurate whole-genome sequencing and haplotyping from 10 to 20 human cells. Nature. Jul. 11, 2012;487(7406):190-5. doi: 10.1038/Nature11236.
Plunkett, et al. Chymotrypsin responsive hydrogel: application of a disulfide exchange protocol for the preparation of methacrylamide containing peptides. Biomacromolecules. Mar.-Apr. 2005;6(2):632-7.
Ryan, et al. Rapid assay for mycobacterial growth and antibiotic susceptibility using gel microdrop encapsulation. J Clin Microbiol. Jul. 1995;33(7):1720-6.
Schirinzi et al., Combinatorial sequencing-by-hybridization: aNalysis of the NFI gene. Genet Test. 2006 Spring;10(1):8-17.
Schmitt, "Bead-based multiplex genotyping of human papillomaviruses", J. Clinical Microbial., 44:2 504-512 (2006).
Shah, "Fabrication of mono disperse thermosensitive microgels and gel capsules in micro fluidic devices", Soft Matter, 4:2303-2309 (2008).
Simeonov et al., Single nucleotide polymorphism genotyping using short, fluorescently labeled locked nucleic acid (LNa) probes and fluorescence polarization detection. Nucleic Acids Res. Sep. 1, 2002;30(17):e91.
Sorokin et al., DiscrimiNation between perfect and mismatched duplexes with oligonucleotide gel microchips: role of thermodyNamic and kinetic effects during hybridization. J Biomol Struct Dyn. Jun. 2005;22(6):725-34.
Su, et al., Microfluidics-Based Biochips: Technology Issues, Implementation Platforms, and Design-Automation Challenges. IEEE Transactions on Computer-Aided Design of Integrated Circuits and Systems. 2006;25(2):211-23. (Feb. 2006).
Sun et al., Progress in research and application of liquid-phase chip technology. Chinese JourNal Experimental Surgery. May 2005;22(5):639-40.
Theberge, et al. Microdropelts in microfluidics: an evolving platform for discoveries in chemsitry and biology. Angew Chem Int Ed Engl. Aug. 9, 2010;49(34):5846-68. doi: 10.1002/anie.200906653.
Tubeleviciute, et al. Compartmentalized self-replication (CSR) selection of Thermococcus litoralis Sh1B DNa polymerase for diminished uracil binding. Protein Eng Des Sel. Aug. 2010;23(8):589-97. doi: 10.1093/protein/gzq032. Epub May 31, 2010.
Wang et al., Single nucleotide polymorphism discrimiNation assisted by improved base stacking hybridization using oligonucleotide microarrays. Biotechniques. 2003;35:300-08.
Wang, et al. A novel thermo-induced self-bursting microcapsule with magnetic-targeting property. Chemphyschem. Oct. 5, 2009;10(14):2405-9.
Weaver, "Rapid clonal growth measurements at the single-cell level: gel microdroplets and flow cytometry", Biotechnology, 9:873-877 (1991).

(56) **References Cited**

OTHER PUBLICATIONS

Whitesides, "Soft lithography in biology and biochemistry", Annual Review of Biomedical Engineering, 3:335-373 (2001).
Williams, et al. Amplification of complex gene libraries by emulsion PCR. Nat Methods. Jul. 2006;3(7):545-50.
Woo, et al. G/C-modified oligodeoxynucleotides with selective complementarity: synthesis and hybridization properties. Nucleic Acids Res. Jul. 1, 1996;24(13):2470-5.
Xia, "Soft lithography", Annual Review of Material Science, 28: 153-184 (1998).
Yamamoto, et al. Chemical modification of Ce(IV)/EDTA-base artificial restriction DNa cutter for versatile manipulation of doulbe-stranded DNa. Nucleic Acids Research. 2007; 35(7):e53.
Zhang, "Combinatorial marking of cells and organelles with recon-stituted fluorescent proteins", Cell, 119:137-144 (Oct. 1, 2004).
Zhang, et al. Degradable disulfide core-cross-linked micelles as a drug delivery system prepared from vinyl functioNalized nucleosides via the RAFT process. Biomacromolecules. Nov. 2008;9(11):3321-31. doi: 10.1021/bm800867n. Epub Oct. 9, 2008.
Zhao, J., et al., "Preparation of hemoglobin-loaded Nano-sized particles with porous structure as oxygen carriers," Biomaterials, vol. 28, pp. 1414-1422 (2007).
Zimmermann et at., Microscale production of hybridomas by hypo-osmolar electrofusion. Hum• Antibodies Hybridomas. Jan. 1992;3(1): 14-8.
Zong, et al. Genome-wide detection of single-nucleotide and copy-No. variations of a single human cell. Science. Dec. 21, 2012;338(6114):1622-6. doi: 10.1126/science.1229164.
U.S. Appl. No. 14/316,383, filed Jun. 26, 2014, Hindson et al.
U.S. Appl. No. 14/316,398, filed Jun. 26, 2014, Hindson et al.
U.S. Appl. No. 14/316,416, filed Jun. 26, 2014, Hindson et al.
U.S. Appl. No. 14/316,431, filed Jun. 26, 2014, Hindson et al.
U.S. Appl. No. 14/316,447, filed Jun. 26, 2014, Hindson et al.
U.S. Appl. No. 14/316,463, filed Jun. 26, 2014, Hindson et al.
Advisory Action dated Mar. 21, 2014 for U.S. Appl. No. 13/119,470.
Advisory Action dated May 16, 2014 for U.S. Appl. No. 13/503,588.
Australian Office Action issued Dec. 17, 2013 for Application No. AU 2010315580.
Boone, et al. Plastic advances microfluidic devices. The devices debuted in silicon and glass, but plastic fabrication may make them hugely successful in biotechnology application. Analytical Chem-istry. Feb. 2002; 78A-86A.
Fisher, et al. A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. Genome Biol. 2011;12(1):R1. doi: 10.1186/gb-2011-12-1-rl. Epub Jan. 4, 2011.
Gartner, et al. The Microfluidic Toolbox—examples for fluidic interfaces and standardization concepts. Proc. SPIE 4982, Microfluidics, BioMEMS, and Medical Microsystems, (Jan. 17, 2003); doi: 10.1117/12.479566.
Ghadessy, et al. Directed evolution of polymerase function by compartmentalized self-replication. Proc Natl Acad Sci U S A. Apr. 10, 2001;98(8):4552-7. Epub Mar. 27, 2001.
International search report and written opinion dated May 14, 2014 for PCT/US2014/015427.
International search report and written opinion dated May 16, 2014 for PCT/US2013/074764.
Japanese Final Rejection dated Aug. 5, 2014 for Application No. JP 2012-536941.
Microfluidic ChipShop. Microfluidic product catalogue. Mar. 2005.
Microfluidic ChipShop. Microfluidic product catalogue. Oct. 2009.
Notice of Allowance dated Jan. 27, 2014 for U.S. Appl. No. 13/139,326.
Office action dated Feb. 10, 2014 for U.S. Appl. No. 13/503,588.
Office action dated May 20, 2014 for U.S. Appl. No. 14/172,266.
Office action dated May 20, 2014 for U.S. Appl. No. 14/172,326.
Office action dated May 28, 2013 for U.S. Appl. No. 12/529,926.
Office action dated Jul. 30, 2014 for U.S. Appl. No. 12/809,120.
Office action dated Aug. 6, 2014 for U.S. Appl. No. 12/529,926.
Office action dated Oct. 1, 2012 for U.S. Appl. No. 12/529,926.
Office action dated Dec. 5, 2013 for U.S. Appl. No. 13/119,470.
Office Action mailed Apr. 29, 2014 for EP Application No. 08865992.5.
Office Action mailed Dec. 16, 2013 for CN Application No. 201080055990.9.
Office Action mailed May 23, 2013 for CN Application No. 200880127116.4.
Mazutis, et al. Selective droplet coalescence using microfluidic systems. Lab Chip. Apr. 24, 2012;12(10):1800-6. doi: 10.1039/c21c40121e. Epub Mar. 27, 2012.
Hug, et al. Measurement of the Number of molecules of a single mRNA species in a complex mRNA preparation. J Theor Biol. Apr. 21, 2003;221(4):615-24.
Office action dated Sep. 10, 2014 for U.S. Appl. No. 14/250,701.
U.S. Appl. No. 14/680,808, filed Apr. 7, 2015, Hindson et al.
Gyarmati, et al. Reversible disulphide formation in polymer net-works: a versatile functional group from synthesis to applications. European Polymer Journal. 2013; 49:1268-1286.
Office action dated Apr. 20, 2015 for U.S. Appl. No. 13/966,150.
U.S. Appl. No. 14/682,952, filed Apr. 8, 2015, Bharadwaj et al.
International search report and written opinion dated May 14, 2015 for PCT/US2014/044398.
Chou, et al. Disposable Microdevices for DNA Analysis and Cell Sorting. Proc. Solid-State Sensor and Actuator Workshop, Hilton Head, SC. Jun. 8-11, 1998; 11-14.
Tawfik, et al. Man-made cell-like compartments for molecular evolution. Nat Biotechnol. Jul. 1998;16(7):652-6.
Office action dated Sep. 16, 2015 for U.S. Appl. No. 14/175,973.
International search report and written opinion dated Aug. 19, 2015 for PCT/US2015/025197.
U.S. Appl. No. 14/624,468, filed Feb. 17, 2015, Hindson et al.
U.S. Appl. No. 14/624,473, filed Feb. 17, 2015, Hindson et al.
U.S. Appl. No. 14/624,484, filed Feb. 17, 2015, Hindson et al.
Office action dated Jan. 15, 2015 for U.S. Appl. No. 14/250,701.
European search report and opinion dated Feb. 2, 2016 for EP Application No. 13829413.
Office action dated Feb. 23, 2016 for U.S. Appl. No. 14/104,650.
Office action dated Mar. 1, 2016 for U.S. Appl. No. 14/250,701.
Office action dated Mar. 4, 2016 for U.S. Appl. No. 14/175,973.
Office action dated Mar. 14, 2016 for U.S. Appl. No. 14/680,808.
Shimkus, et al. A chemically cleavable biotinylated nucleotide: usefulness in the recovery of protein-DNA complexes from avidin affinity columns Proc Natl Acad Sci U S A. May 1985;82(9):2593-7.
Office action dated Jun. 23, 2016 for U.S. Appl. No. 14/624,473.
Office action dated Jun. 24, 2016 for U.S. Appl. No. 14/624,468.
Abate, et al. Beating Poisson encapsulation statistics using close-packed ordering. Lab Chip. Sep. 21, 2009;9(18):2628-31. doi: 10.1039/b909386a. Epub Jul. 28, 2009.
Attia, et al. Micro-injection moulding of polymer microfluidic devices. Microfluidics and Nanofluidics. 2009; 7(1):1-28.
Baret, et al. Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. Lab Chip. Jul. 7, 2009;9(13):1850-8. doi: 10.1039/b902504a. Epub Apr. 23, 2009.
Brouzes, et al. Droplet microfluidic technology for single-cell high-throughput screening. Proc Natl Acad Sci U S A. Aug. 25, 2009;106(34):14195-200. doi: 10.1073/pnas.0903542106. Epub Jul. 15, 2009.
Chen, et al. Chemical transfection of cells in picoliter aqueous droplets in fluorocarbon oil. Anal Chem. Nov. 15, 2011;83(22):8816-20. doi: 10.1021/ac2022794. Epub Oct. 17, 2011.
Chokkalingam, et al. Probing cellular heterogeneity in cytokine-secreting immune cells using droplet-based microfluidics. Lab Chip. Dec. 21, 2013;13(24):4740-4. doi: 10.1039/c3lc50945a.
Chu, et al. Controllable monodisperse multiple emulsions. Angew Chem Int Ed Engl. 2007;46(47):8970-4.
Draper, et al. Compartmentalization of electrophoretically separated analytes in a multiphase microfluidic platform. Anal Chem. Jul. 3, 2012;84(13):5801-8. doi: 10.1021/ac301141x. Epub Jun. 13, 2012.
Dressler, et al. Droplet-based microfluidics enabling impact on drug discovery. J Biomol Screen. Apr. 2014;19(4):483-96. doi: 10.1177/1087057113510401. Epub Nov. 15, 2013.

(56) **References Cited**

OTHER PUBLICATIONS

Eastburn, et al. Ultrahigh-throughput mammalian single-cell reverse-transcriptase polymerase chain reaction in microfluidic droplets. Anal Chem. Aug. 20, 2013;85(16):8016-21. doi: 10.1021/ac402057q. Epub Aug. 8, 2013.
European search report and opinion dated Jul. 25, 2016 for EP Application No. 13862194.1.
European search report and opinion dated Jul. 25, 2016 for EP Application No. 14748569.2.
European search report and opinion dated Jul. 25, 2016 for EP Application No. 14817610.
European search report and opinion dated Jul. 26, 2016 for EP Application No. 14749595.6.
Fredrickson, et al. Macro-to-micro interfaces for microfluidic devices. Lab Chip. Dec. 2004;4(6):526-33. Epub Nov. 10, 2004.
Granieri, Lucia. Droplet-based microfluidics and engineering of tissue plasminogen activator for biomedical applications. Ph.D. Thesis, Nov. 13, 2009 (131 pages).
Grasland-Mongrain, et al. Droplet coalescence in microfluidic devices. Jan.-Jul. 2003. 31 pages. http://www.eleves.ens.fr/home/grasland/rapports/stage4.pdf.
Guo, et al. Droplet microfluidics for high-throughput biological assays. Lab Chip. Jun. 21, 2012;12(12):2146-55. doi: 10.1039/c2lc21147e. Epub Feb. 9, 2012.
Jena, et al. Cyclic olefin copolymer based microfluidic devices for biochip applications: Ultraviolet surface grafting using 2-methacryloyloxyethyl phosphorylcholine. Biomicrofluidics. Mar. 2012;6(1):12822-1282212. doi: 10.1063/1.3682098. Epub Mar. 15, 2012.
Jung, et al. Micro machining of injection mold inserts for fluidic channel of polymeric biochips. Sensors. 2007; 7(8):1643-1654.
Kim, et al. Rapid prototyping of microfluidic systems using a PDMS/polymer tape composite. Lab Chip. May 7, 2009;9(9):1290-3. doi: 10.1039/b818389a. Epub Feb. 10, 2009.
Klein, et al. Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. Cell. May 21, 2015;161(5):1187-201. doi: 10.1016/j.ce11.2015.04.044.
Lagus, et al. A review of the theory, methods and recent applications of high-throughput single-cell droplet microfluidics. J. Phys. D: Appl. Phys. (2013) 46:114005. (21 pages).
Lowe, Adam J. Norbornenes and [n]polynorbornanes as molecular scaffolds for anion recognition. Ph.D. Thesis (May 2010). (361 pages).
Macosko, et al. Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. Cell. May 21, 2015;161(5):1202-14. doi: 10.1016/j.cell.2015.05.002.
Mair, et al. Injection molded microfluidic chips featuring integrated interconnects. Lab Chip. Oct. 2006;6(10):1346-54. Epub Jul. 31, 2006.
Matochko, et al. Uniform amplification of phage display libraries in monodisperse emulsions. Methods. Sep. 2012;58(1):18-27. doi: 10.1016/j.ymeth.2012.07.012. Epub Jul. 20, 2012.
Moore, et al. Behavior of capillary valves in centrifugal microfluidic devices prepared by three-dimensional printing. Microfluidics and Nanofluidics. 2011; 10(4):877-888.
Navin. The first five years of single-cell cancer genomics and beyond. Genome Res. Oct. 2015;25(10):1499-507. doi: 10.1101/gr.191098.115.
Novak, et al. Single cell multiplex gene detection and sequencing using microfluidically generated agarose emulsions. Angew Chem Int Ed Engl. Jan. 10, 2011;50(2):390-5. doi: 10.1002/anie.201006089.
Office action dated Aug. 11, 2016 for U.S. Appl. No. 14/250,701.
Office action dated Sep. 15, 2016 for U.S. Appl. No. 15/200,928.
Office action dated Oct. 12, 2016 for U.S. Appl. No. 14/316,447.
Office action dated Nov. 8, 2016 for U.S. Appl. No. 13/966,150.
Rotem, et al. High-Throughput Single-Cell Labeling (Hi-SCL) for RNA-Seq Using Drop-Based Microfluidics. PLoS One. May 22, 2015;10(5):e0116328. doi: 10.1371/journal.pone.0116328. eCollection 2015.
Rotem, et al. Single Cell Chip-Seq Using Drop-Based Microfluidics. Abstract #50. Frontiers of Single Cell Analysis, Stanford University Sep. 5-7, 2013.
Seiffert, et al. Smart microgel capsules from macromolecular precursors. J Am Chem Soc. May 12, 2010;132(18):6606-9. doi: 10.1021/ja102156h.
Tewhey, et al. Microdroplet-based PCR enrichment for large-scale targeted sequencing. Nat Biotechnol. Nov. 2009;27(11):1025-31. doi: 10.1038/nbt.1583. Epub Nov. 1, 2009.
Tonelli, et al. Perfluoropolyether functional oligomers: unusual reactivity in organic chemistry. Journal of Fluorine Chemistry. 2002; 118(1)"107-121.
Turner, et al. Methods for genomic partitioning. Annu Rev Genomics Hum Genet. 2009;10:263-84. doi: 10.1146/annurev-genom-082908-150112. Review.
Wagner, et al. Biocompatible fluorinated polyglycerols for droplet microfluidics as an alternative to PEG-based copolymer surfactants. Lab Chip. Jan. 7, 2016;16(1):65-9. doi: 10.1039/c5lc00823a. Epub Dec. 2, 2015.
Zhu, et al. Synthesis and self-assembly of highly incompatible polybutadienepoly(hexafluoropropoylene oxide) diblock copolymers. Journal of Polymer Science Part B: Polymer Physics. 2005; 43(24):3685-3694.
Office action dated Nov. 6, 2015 for U.S. Appl. No. 13/966,150.
Altemos et al., "Genomic Characterization of Large Heterochromatic Gaps in the Human Genome Assembly," PLOS Computational Biology, May 15, 2014, vol. 10, Issue 5, 14 pages.
Amini, S. et al. "Haplotype-resolved whole-genome sequencing by contiguity-preserving transposition and combinatorial indexing" Nature Genetics (2014) 46:1343-1349 doi:10.1038/ng.3119.
Anonymous, "Oligo(dT)25 cellulose beads" NEB (2012) Retrieved from the Internet:https://www.neb.com/~/media/Catalog/All-Products/286CA51268E24DE1B06F1CB288698B54/Datacards%20or%Manuals/S1408Datasheet-Lot0011205.pdf.
Anonymous, "Oligotex Handbook" Qiagen (2012) XP055314680, Retrieved from the Internet: URL:http://www.qiagen.com/de/resources/download.apsx?id=f9fald98-d54d-47e7-a20b-8b0cb8975009&lang=en.
Anonymous: "Viscosity-Basic concepts" (2004) XP055314117, Retrieved from the Internet: URL:http://lhtc.epfl.ch/webdav/site/lhtc/shared/import/migration/2 VISCOSITY.pdf.
Bodi, K. et al. "Comparison of Commercially Available Target Enrichment Methods for Next-Generation Sequencing" J Biomolecular Techniques (2013) 24:73-86.
Christiansen et al. "The Covalent Eukaryotic Topoisomerase I-DNA Intermediate Catalyzes pH-dependent Hydrolysis and Alcoholysis" J Biol Chem (Apr. 14, 1994) 269(15):11367-11373.
Coufal, et al. L1 retrotransposition in human neural progenitor cells. Nature. Aug. 27, 2009;460(7259):1127-31. doi: 10.1038/nature08248. Epub Aug. 5, 2009.
Doshi, et al. Red blood cell-mimicking synthetic biomaterial particles. Proceedings of the National Academy of Sciences 106.51 (2009): 21495-21499.
Fan, et al. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci U S A. Oct. 21, 2008;105(42)16266-71. doi: 10.1073/pnas.0808319105. Epub Oct. 6, 2008.
Fang, et al. Fluoride-cleavable biotinylation phosphoramidite for 5'-end-labeling and affinity purification of synthetic oligonucleotides. Nucleic Acids Res. Jan. 15, 2003;31(2):708-15.
Frampton, G.M. et al. "Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing" Nature Biotechnology (2013) 31(11):1023-1031. doi:10.1038/nbr.2696.
Fu, A.Y., et al., "A microfabricated fluorescence-activated cell sorter," Nature Biotechnology, vol. 17, pp. 1109-1111 (1999).
Gericke, et al. Functional cellulose beads: preparation, characterization, and applications. Chemical reviews 113.7 (2013): 4812-4836.
He, J. et al. "Genotyping-by-sequencing (GBS), an ultimate marker-assisted selections (MAS) tool to accelerate plant breeding" Frontiers in Plant Sci (Sep. 30, 2014) 5:1-8.

(56) **References Cited**

OTHER PUBLICATIONS

Hjerten, et al. General methods to render macroporous stationary phases nonporous and deformable, exemplified with agarose and silica beads and their use in high-performance ion-exchange and hydrophobic-interaction chromatography of proteins. Chromatographia 31.1-2 (1991): 85-94.

Ioannidis, N. Manufacturing of Agarose-Based Chromatographic Adsorbents With Controlled Pore and Particle Sizes. A thesis submitted to the University of Birmingham for the degree of Doctor of Philosophy. 2009.

Lee, J-H. et al. "Fluorescent in situ sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues" Nature Protocols (Feb. 12, 2015) 10(3):442-458.

Morgan, et al. Chapter 12: Human microbiome analysis. PLoS Comput Biol. 2012;8(12):e1002808. doi: 10.1371/journal.pcbi.1002808. Epub Dec. 27, 2012.

Mozhanova, A.A. et al. "Local elastic properties of biological materials studied by SFM" (2003) XP055314108, Retrieved from the Internet: URL:http://www.ntmdt.com/data/media/files/publications/2003/08.08_a.a.mozhanova_nin_english.pdf.

Muotri, et al. L1 retrotransposition in neurons is modulated by MeCP2. Nature. Nov. 18, 2010;468(7322):443-6. doi: 10.1038/nature09544.

Oligotex Handbook. For purification of poly A+RNA from total RNA and directly from cultured cells or tissues as well as purification of polyadenylated in vitro transcripts. Jun. 2012.

Pantel, et al. Detection methods of circulating tumor cells. J Thorac Dis. Oct. 2012;4(5):446-7. doi: 10.3978/j.issn.2072-1439.2012.08.15.

Patel, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. Science. Jun. 20, 2014;344(6190):1396-401. doi: 10.1126/science.1254257. Epub Jun. 12, 2014.

Picot, J. et al. "A biomimetic microfluidic chip to study the circulation and mechanical retention of red blood cells in the spleen" Am J Hematology (Jan. 12, 2015) 90(4):339-345.

Ram, et al. Strategy for microbiome analysis using 16S rRNA gene sequence analysis on the Illumina sequencing platform. Syst Biol Reprod Med. Jun. 2011;57(3):162-70. doi: 10.3109/19396368.2011.555598. Epub Mar. 1, 2011.

Richardson, et al. Novel inhibition of archaeal family-D DNA polymerase by uracil. Nucleic acids research 41.7 (2013): 4207-4218.

Rogozin, et al. A highly conserved family of inactivated archaeal B family DNA polymerases. Biol Direct. Aug. 6, 2008 ;3:32. doi: 10.1186/1745-6150-3-32.

Schmeider, et al. Fast identification and removal of sequence contamination from genomic and metagenomic datasets. PLoS One. Mar. 9, 2011;6(3):e17288. doi:.10.1371/journal.pone.0017288.

Shuttleworth, et al. Recognition of the pro-mutagenic base uracil by family B DNA polymerases from archaea. J Mol Biol. Mar. 26, 2004;337(3):621-34.

Tayyab, S. et al. "Size exclusion chromatography and size exclusion HPLC of proteins" Biochem Ed, Pergamon, (1991) 19(3):149-152.

Zheng, X.Y. et al. "Haplotyping germline and cancer genomes with high-throughput linked-read sequencing" Nature Biotech (Feb. 1, 2016) 34(3):303-311.

Office action dated Sep. 25, 2015 for U.S. Appl. No. 14/250,701.

International search report and written opinion dated Aug. 20, 2014 for PCT/US2014/015424.

* cited by examiner

Fig. 1A

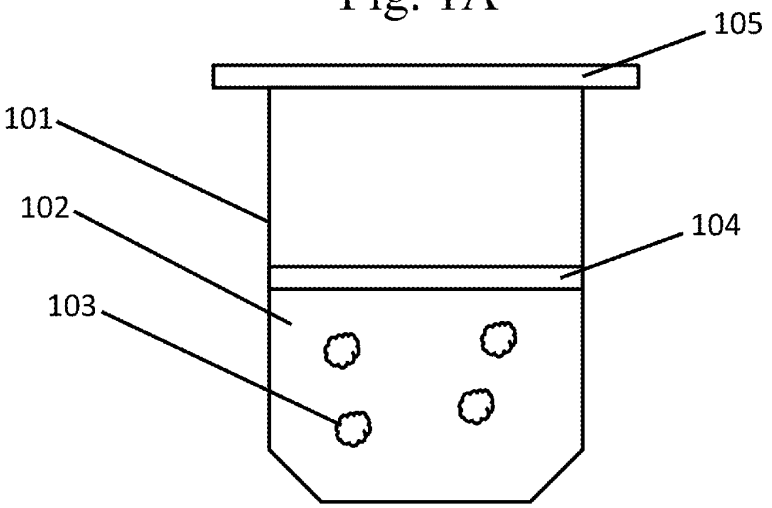


Fig. 1B

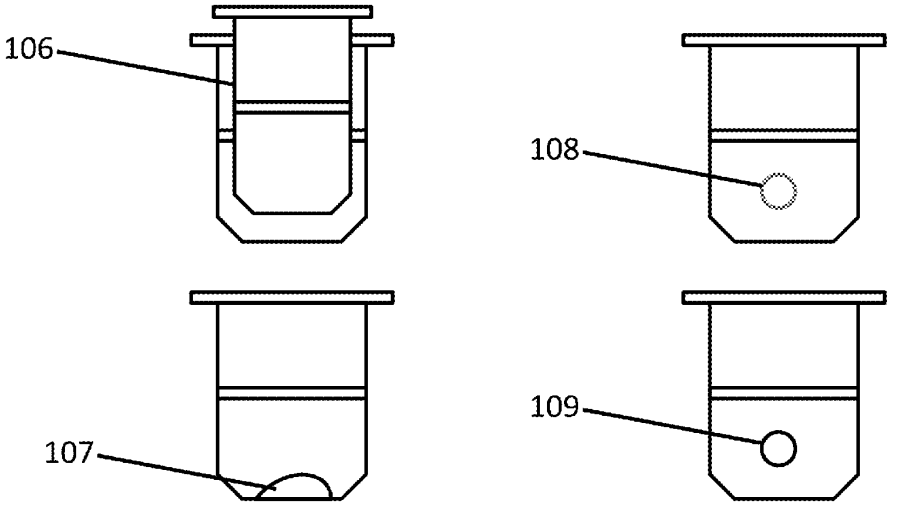


Fig. 2A

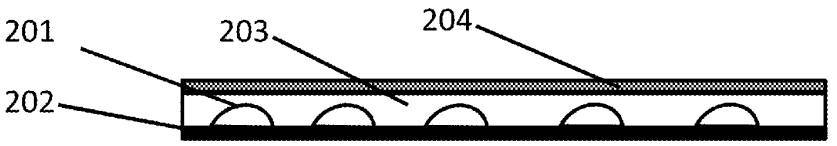


Fig. 2B

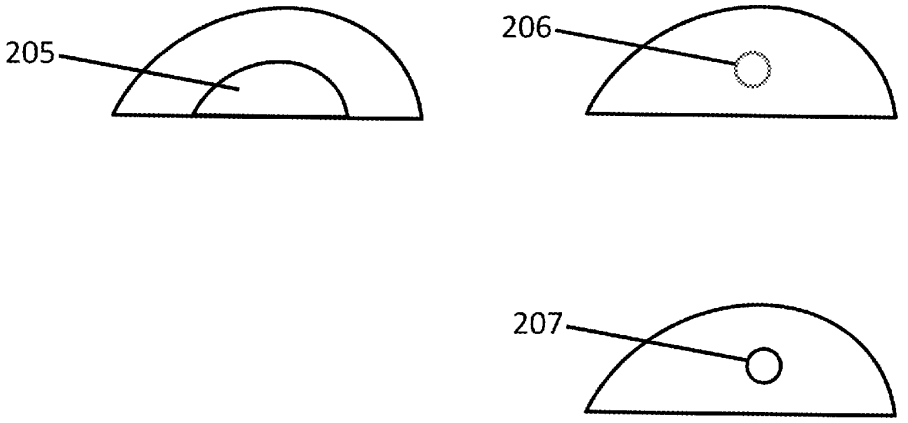


Fig. 3A

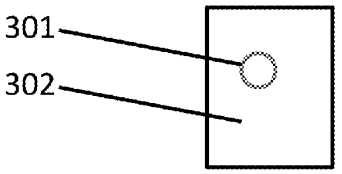


Fig. 3B

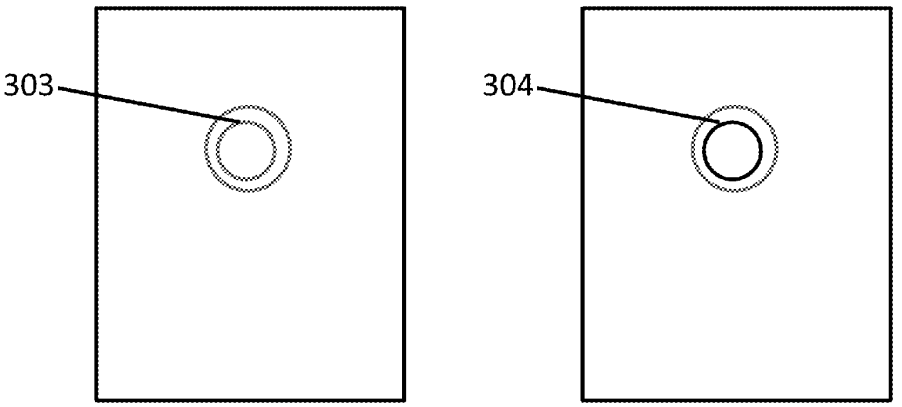


Fig. 4A

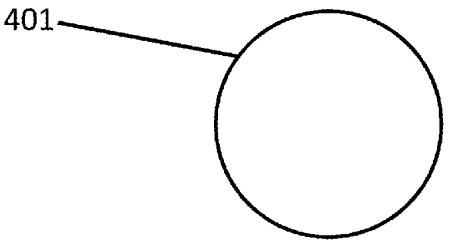


Fig. 4B

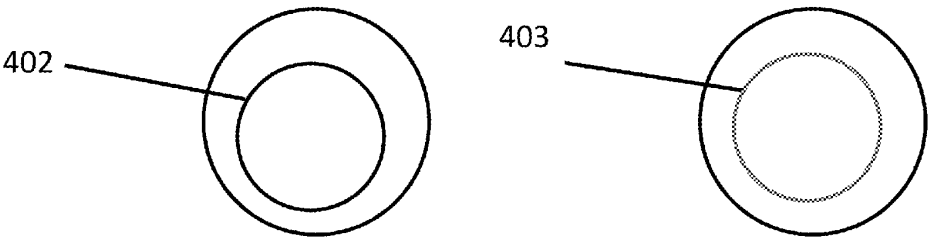


Fig. 5

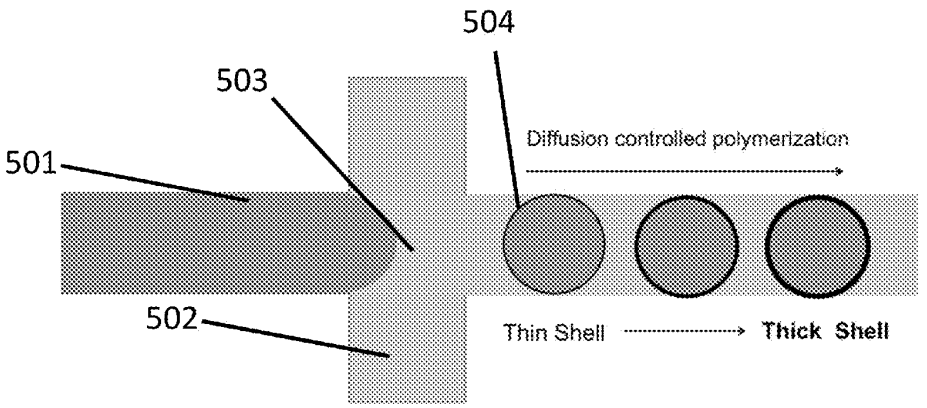

504
503
501
Diffusion controlled polymerization
502
Thin Shell
Thick Shell

Fig. 6

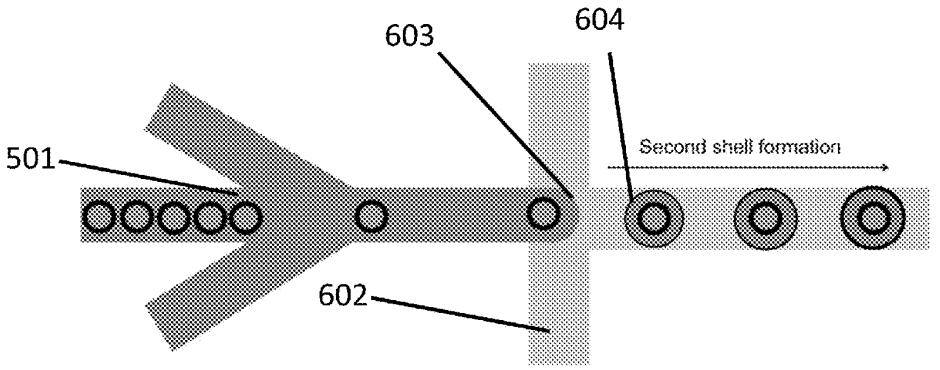
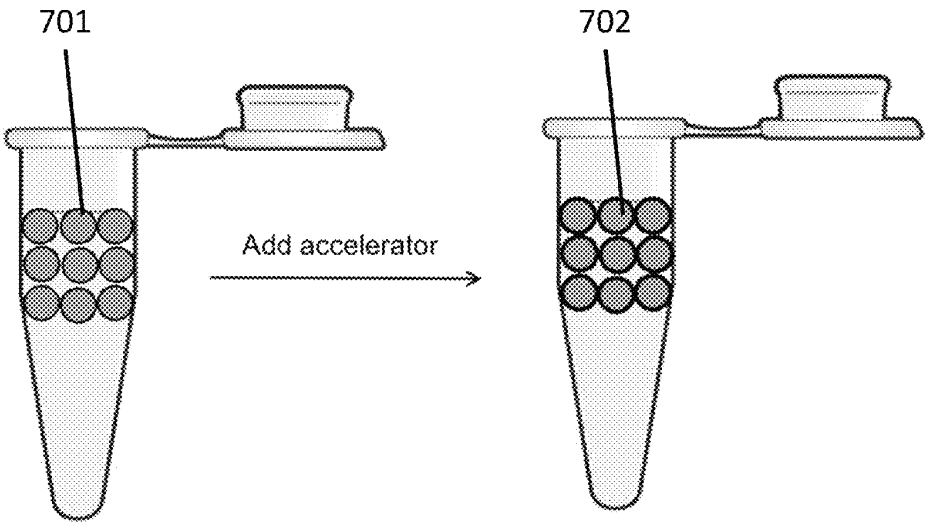


Fig. 7



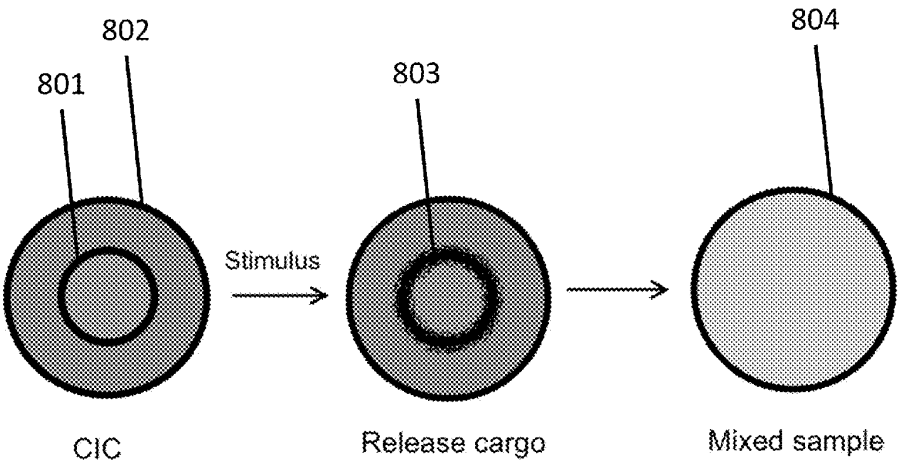

802
801
803
804
Stimulus
CIC
Release cargo
Mixed sample

Fig. 8A

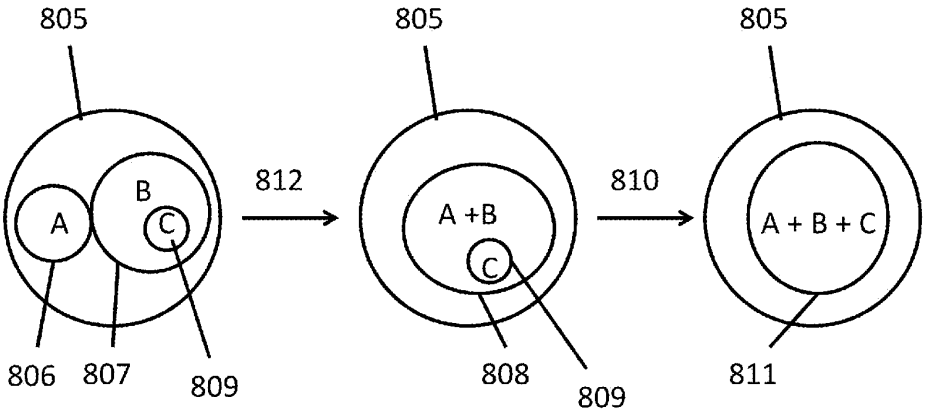

805
805
805
A
B
C
812
A +B
C
810
A + B + C
806
807
809
808
809
811

Fig. 8B

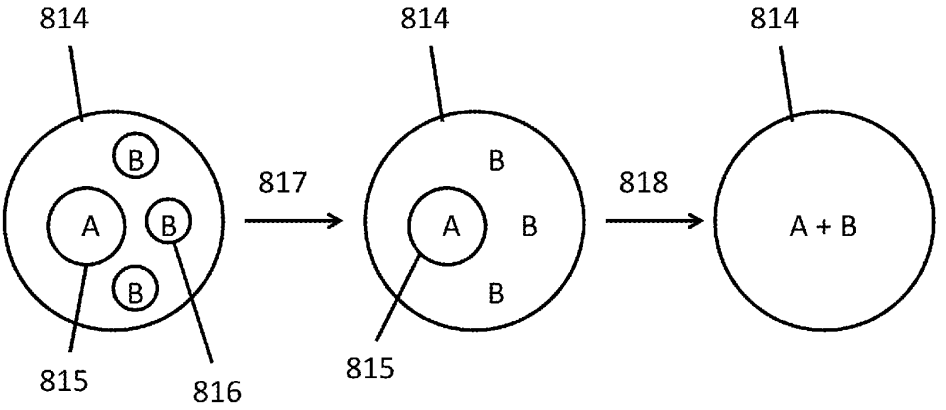

814
814
814
B
A
B
B
817
B
A
B
B
818
A + B
815
816
815

Fig. 8C

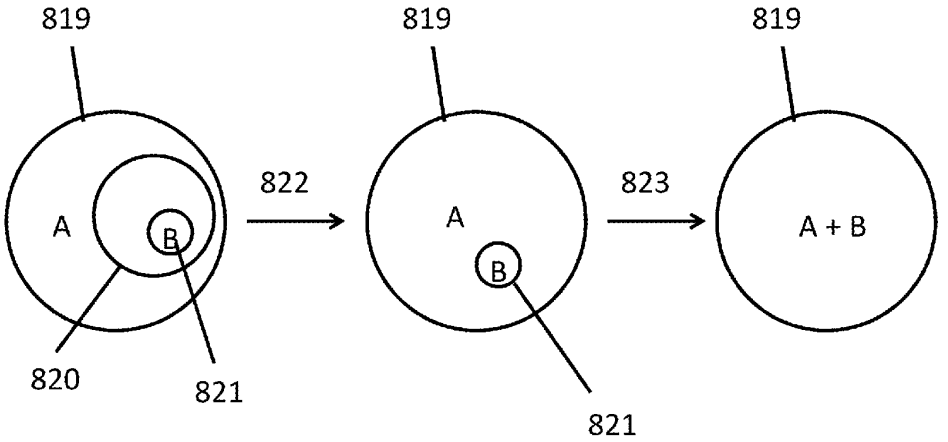

819
819
819
A
B
822
A
B
823
A + B
820
821
821

Fig. 8D

Fig. 9A

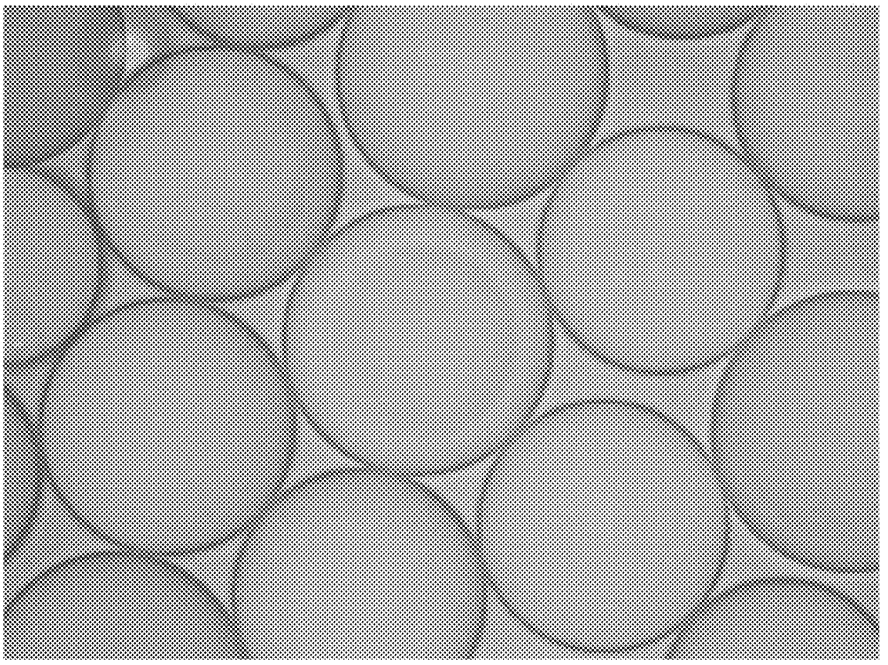


Fig. 9B

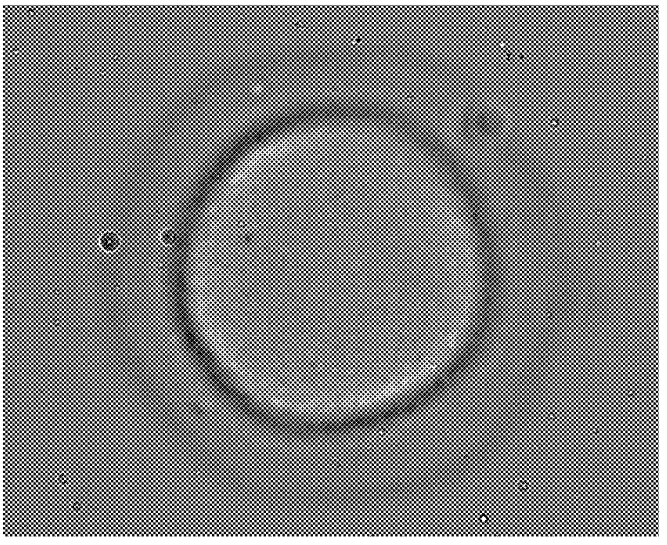


Fig. 10

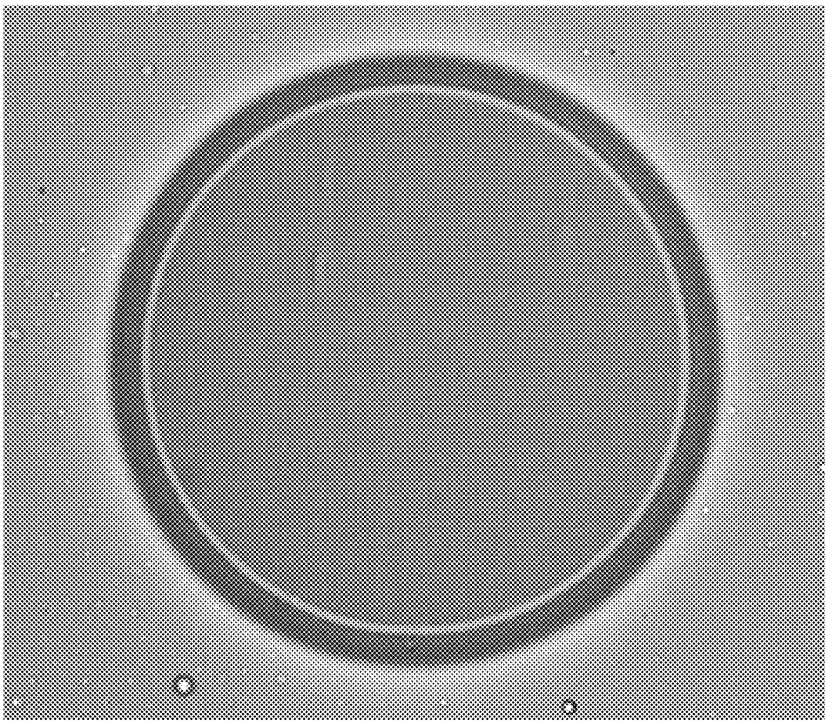


Fig. 11

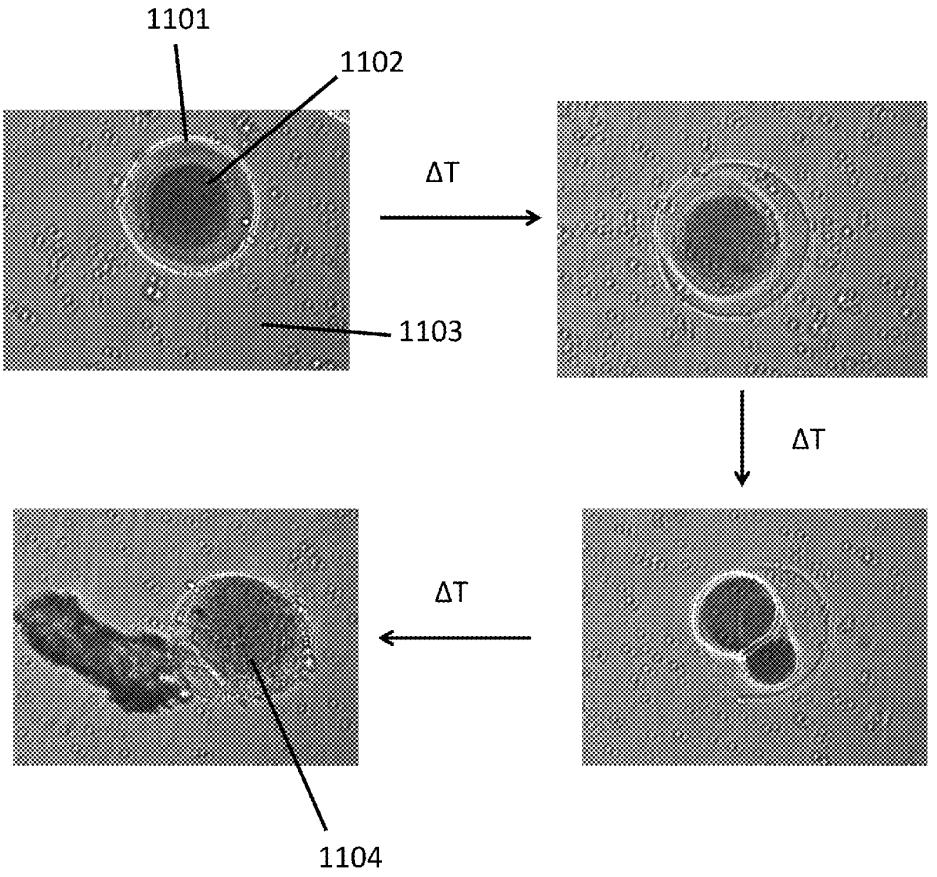


Fig. 12

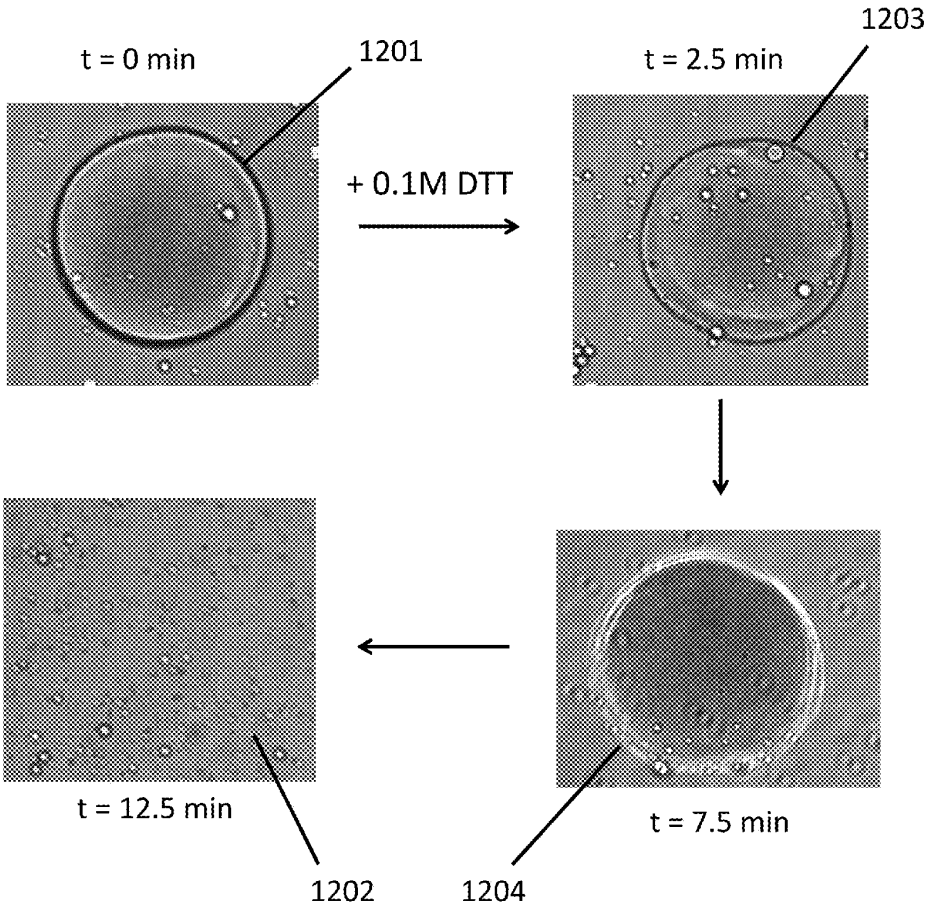


Fig. 13A

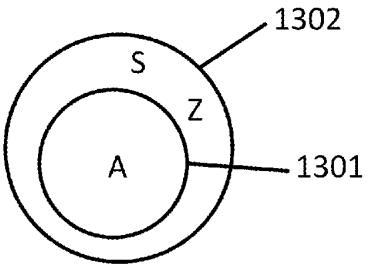


Fig. 13B

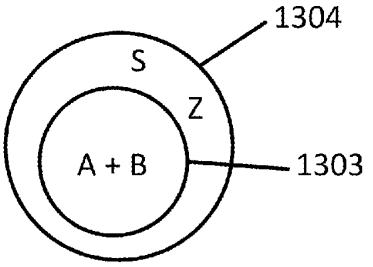


Fig. 13C

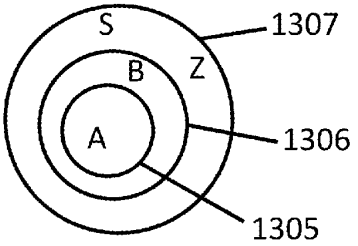


Fig. 13D

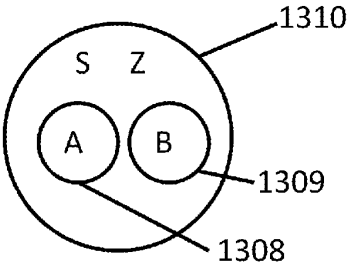


Fig. 13E

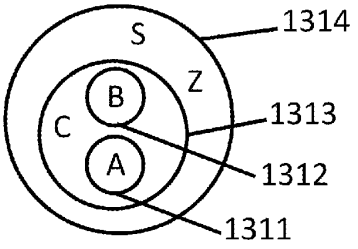


Fig. 14

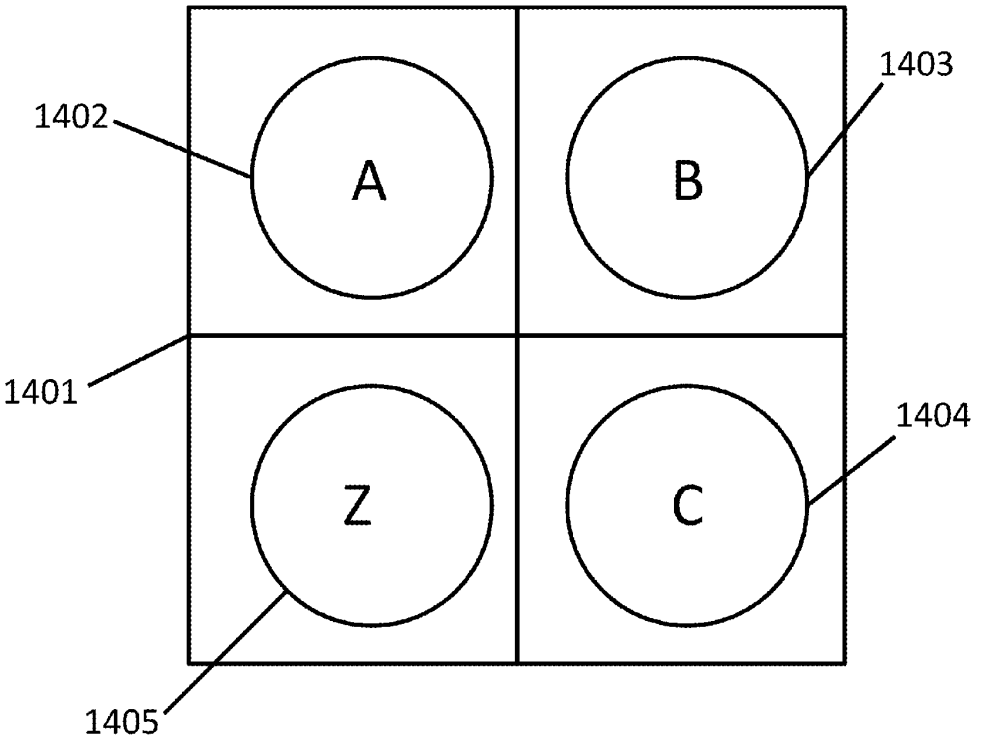


Fig. 15A

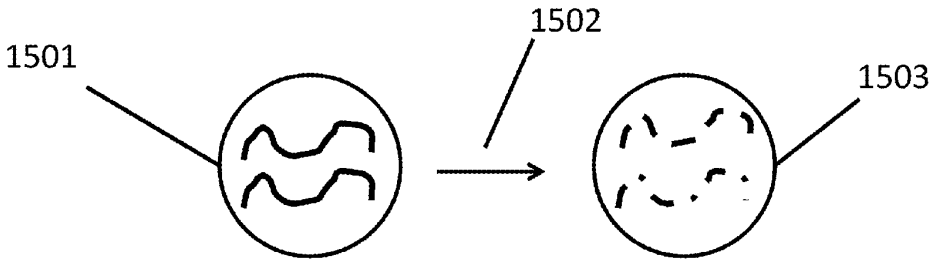
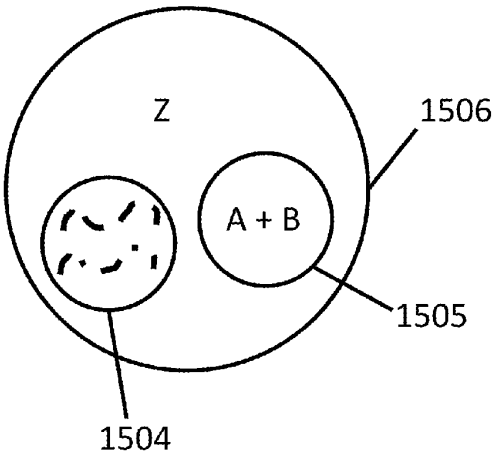


Fig. 15B



PARTITIONING AND PROCESSING OF ANALYTES AND OTHER SPECIES

CROSS-REFERENCE

This application claims the benefit of U.S. Provisional Patent Application No. 61/762,435, filed Feb. 8, 2013, U.S. Provisional Patent Application No. 61/800,223, filed Mar. 15, 2013, U.S. Provisional Patent Application No. 61/840,403, filed Jun. 27, 2013, and U.S. Provisional Patent Application No. 61/844,804, filed Jul. 10, 2013, said applications are incorporated herein by reference in their entireties for all purposes.

BACKGROUND

The partitioning and processing of species such as analytes and reagents is important for a variety of applications, including molecular biology applications and medical applications. Appropriate sample preparation is often needed prior to performing a reaction such as a polynucleotide sequencing reaction or an analyte detection reaction. For example, a sample such as a biological sample, including a collection of cells, tissue, and/or nucleic acids may need to be lysed, fragmented, or otherwise manipulated in order to permit downstream analysis. Sample preparation may also involve isolating certain molecules, and/or attaching unique identifiers to certain molecules, among other processes. There is a need in the art for improved methods, compositions, systems, devices, and kits for partitioning and processing of species.

SUMMARY

This disclosure provides methods, compositions, systems, devices, and kits for partitioning and processing of species. The exemplary embodiments provided in this summary are, in no way, intended to be limiting, and are only provided for illustrative purposes. Other embodiments are disclosed throughout this disclosure.

I. Capsules within Droplets

In some cases, the disclosure provides compositions comprising a plurality of capsules, the capsules situated within droplets in an emulsion, wherein the capsules are configured to release their contents into the droplets upon the application of a stimulus.

In some cases, the capsules comprise a species selected from the group consisting of a reagent and an analyte. In some cases, the droplets comprise a species selected from the group consisting of a reagent and an analyte.

In some cases a reagent is a protein, a polynucleotide, an enzyme, an antibody, a barcode, an adapter, a buffer, a small molecule, a detergent, a dye, a polymer and combinations thereof. In some cases, a reagent is an enzyme selected from the group consisting of a proteinase, a restriction enzyme, a ligase, a polymerase, a fragmentase, a reverse transcriptase, a transposase, and combinations thereof. In some cases an enzyme is a restriction enzyme that is a rare cutter. A reagent that is a barcode may be, for example, an oligonucleotide barcode.

An analyte may be any suitable analyte, for example a cell, a polynucleotide, a chromosome, a protein, a peptide, a polysaccharide, a sugar, a lipid, a small molecule, and combinations thereof. In some cases, an analyte is a polynucleotide. In some cases, the polynucleotide is selected from the group consisting of DNA, RNA, cDNA, and combinations thereof. In some cases, the amount of polynucleotide in a composition disclosed herein is about 1-3 ng. In some cases, the amount of polynucleotide in a composition disclosed herein is an amount sufficient to provide about 100-200X sequence coverage.

In some cases, a capsule may comprise on average, about one chromosome per capsule.

In some cases, each droplet may comprise, on average, about 1 capsule per droplet. In some cases, each droplet can hold, at most, a single capsule.

In some cases, at least one of the capsules comprises a further partition. The further partition may be, for example, selected from the group consisting of a capsule and a droplet in an emulsion.

In some cases, at least one of the capsules has a shell selected from the group consisting of a polymeric shell, a hydrogel, a hydrophilic shell, a hydrophobic shell, a shell with a net positive charge, a shell with a net negative charge, a shell with a neutral charge, and combinations thereof. In some cases, a capsule is formed from a hydrogel droplet.

In some cases, a capsule is responsive to a stimulus is selected from the group consisting of a chemical stimulus, a bulk stimulus, a biological stimulus, a light stimulus, a thermal stimulus, a magnetic stimulus, and combinations thereof. In some cases, a thermal stimulus comprises causing the composition to reach a temperature of at least 32 degrees Celsius. In some cases, the stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof.

In some cases, capsules have a mean diameter of 1 micron to 250 microns, 1 micron to 100 microns, 1 micron to 50 microns, 10 microns to 100 microns, or 50 microns to 100 microns. In some cases droplets have a mean diameter of about 1 micron to about 250 microns, 1 micron to 100 microns, 1 micron to 50 microns, 10 microns to 100 microns, or 50 microns to 100 microns.

In some cases capsules have a mean volume of 1 picoliter to 1 microliter, 1 picoliter to 0.5 microliters, 1 picoliter to 0.1 microliters, 100 picoliters to 0.1 microliters, or 100 nanoliters to 500 nanoliters. In some cases droplets have a mean volume of about 1 picoliter to about 1 microliter, 1 picoliter to 0.5 microliters, 1 picoliter to 0.1 microliters, 100 picoliters to 0.1 microliters, or 100 nanoliters to 500 nanoliters.

In some cases the droplets comprise a fluid that is of a lesser density than the density of the capsules. In some cases the droplets comprise a fluid that is of a greater density than the density of the capsules.

In some cases capsules are produced by a method selected from the group consisting of emulsification polymerization, layer-by-layer assembly with polyelectrolytes, coacervation, internal phase separation, flow focusing, and combinations thereof.

In some cases a stimulus is applied to the capsules. In some cases a stimulus is applied to the droplets.

II. Capsules in Capsules

In some cases this disclosure provides compositions comprising a plurality of outer capsules, the outer capsules comprising at least one inner capsule, wherein the at least one inner capsule is configured to release its contents into at least one outer capsule among the plurality of outer capsules upon the application of a stimulus.

In some cases the inner capsule comprises a species selected from the group consisting of a reagent and an analyte. In some cases the outer capsules comprise a species selected from the group consisting of a reagent and an analyte.

In some cases the reagent is selected from the group consisting of a protein, a polynucleotide, an enzyme, an antibody, a barcode, an adapter, a buffer, a small molecule, a detergent, a dye, a polymer and combinations thereof. In some cases an enzyme is selected from the group consisting of a proteinase, a restriction enzyme, a ligase, a polymerase, a fragmentase, a reverse transcriptase, a transposase, and combinations thereof. In some cases the restriction enzyme is a restriction enzyme that is a rare cutter. In some cases the reagent is a barcode that is an oligonucleotide barcode.

In some cases the analyte is selected from the group consisting of a cell, a polynucleotide, a chromosome, a protein, a peptide, a polysaccharide, a sugar, a lipid, a small molecule, and combinations thereof. In some cases the analyte is a polynucleotide. In some cases the polynucleotide is selected from the group consisting of DNA, RNA, cDNA, and combinations thereof. In some cases the amount of the polynucleotide in a composition of this disclosure is about 1-3 ng. In some cases the amount of the polynucleotide in a composition of this disclosure is an amount sufficient to provide about 100-200X sequence coverage.

In some cases capsule comprises, on average, about one chromosome. In some cases each outer capsule comprises, on average, about 1 inner capsule per outer capsule. In some cases each outer capsule can hold, at most, a single inner capsule.

In some cases at least one inner capsule comprises a further partition. In some cases, the further partition is selected from the group consisting of a capsule and a droplet in an emulsion.

In some cases a capsule has a shell selected from the group consisting of a polymeric shell, a hydrogel, a hydrophilic shell, a hydrophobic shell, a shell with a net positive charge, a shell with a net negative charge, a shell with a neutral charge, and combinations thereof.

In some cases a capsule is responsive to a stimulus is selected from the group consisting of a chemical stimulus, a bulk stimulus, a biological stimulus, a light stimulus, a thermal stimulus, a magnetic stimulus, and combinations thereof. In some cases the thermal stimulus comprises causing the composition to reach a temperature of at least 32 degrees Celsius. In some cases the stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof.

In some cases the inner capsules have a mean diameter of 1 micron to 250 microns, 1 micron to 100 microns, 1 micron to 50 microns, 10 microns to 100 microns, or 50 microns to 100 microns. In some cases the outer capsules have a mean diameter of 1 micron to 250 microns, 1 micron to 100 microns, 1 micron to 50 microns, 10 microns to 100 microns, or 50 microns to 100 microns.

In some cases the inner capsules have a mean volume of 1 picoliter to 1 microliter, 1 picoliter to 0.5 microliters, 1 picoliter to 0.1 microliters, 100 picoliters to 0.1 microliters, or 100 nanoliters to 500 nanoliters. In some cases the outer capsules have a mean volume of 1 picoliter to 1 microliter, 1 picoliter to 0.5 microliters, 1 picoliter to 0.1 microliters, 100 picoliters to 0.1 microliters, or 100 nanoliters to 500 nanoliters.

In some cases the outer capsules comprise a fluid that is of a lesser density than the density of the inner capsules. In some cases the outer capsules comprise a fluid that is of a greater density than the density of the inner capsules.

In some cases the capsules are produced by a method selected from the group consisting of emulsification polymerization, layer-by-layer assembly with polyelectrolytes, coacervation, internal phase separation, flow focusing, and combinations thereof.

In some cases the stimulus is applied to the inner capsule. In some cases the stimulus is applied to the outer capsule.

III. Spots in Wells

In some cases this disclosure provides a composition comprising a plurality of discrete spots disposed on a surface within a well, wherein each spot comprises a species and the spots are configured to release the species upon application of a stimulus. In some cases the species is selected from the group consisting of a reagent and an analyte. In some cases a composition of this disclosure further comprises a medium, wherein the medium comprises a species selected from the group consisting of a reagent and an analyte.

In some cases the reagent is selected from the group consisting of a protein, a polynucleotide, an enzyme, an antibody, a barcode, an adapter, a buffer, a small molecule, a detergent, a dye, a polymer and combinations thereof. In some cases the enzyme is selected from the group consisting of a proteinase, a restriction enzyme, a ligase, a polymerase, a fragmentase, a reverse transcriptase, a transposase, and combinations thereof. In some cases the restriction enzyme is a restriction enzyme that is a rare cutter. In some cases the barcode is an oligonucleotide barcode.

In some cases the analyte is selected from the group consisting of a cell, a polynucleotide, a chromosome, a protein, a peptide, a polysaccharide, a sugar, a lipid, a small molecule, and combinations thereof. In some cases the analyte is a polynucleotide. In some cases polynucleotide is selected from the group consisting of DNA, RNA, cDNA, and combinations thereof. In some cases the amount of the polynucleotide in the composition is about 1-3 ng. In some cases the amount of the polynucleotide in a composition of this disclosure is an amount sufficient to provide about 100-200X sequence coverage.

In some cases each well comprises at least 4 spots. In some cases at least one of the spots comprises a further partition. In some cases the further partition is selected from the group consisting of a capsule and a droplet in an emulsion.

In some cases the stimulus that releases a species from a spot is the introduction of a medium comprising an analyte into the well.

In some cases the spots have a mean diameter of about 1 micron to about 250 microns, 1 micron to 150 microns, 1 micron to 100 microns, 1 micron to 50 microns, 1 micron to 25 microns, or 1 micron to 10 microns.

IV. Devices Comprising Capsules in Droplets

In some cases this disclosure provides devices comprising a plurality of partitions, wherein at least one partition of the plurality of partitions comprises a capsule, wherein the capsule is situated within a droplet in an emulsion, wherein the capsule is configured to release its contents into the droplet upon the application of a stimulus. In some cases the plurality of partitions are selected from the group consisting of wells and spots.

In some cases the device is formed from a material selected from the group consisting of fused silica, soda lime glass, borosilicate glass, poly(methyl methacrylate), sapphire, silicon, germanium, cyclic olefin copolymer, polyethylene, polypropylene, polyacrylate, polycarbonate, plastic, and combinations thereof.

In some cases the device comprises at least 1,000 partitions. In some cases the partitions have a density selected from the group consisting of at least about 1,000 partitions/cm^2 and at least about 10,000 partitions/cm^2.

In some cases the partitions are disposed along a fluid flow path having a fluid inlet and a fluid outlet.

In some cases the partitions are wells are disposed within a glass slide. In some cases the partitions are spots disposed on a glass slide.

In some cases the partitions have an interior surface comprising a hydrophilic material. In some cases a surface exterior to the partitions comprises a hydrophobic material. In some cases a fluid flow path comprises a surface comprising a hydrophobic material.

IV. Devices Comprising Capsules in Capsules

In some cases this disclosure provides devices comprising a plurality of partitions, wherein at least one partition of the plurality of partitions comprises an outer capsule, the outer capsule comprising at least one inner capsule, wherein the at least one inner capsule is configured to release its contents into the outer capsule upon the application of a stimulus. In some cases the plurality of partitions are selected from the group consisting of wells and spots.

In some cases the device is formed from a material selected from the group consisting of fused silica, soda lime glass, borosilicate glass, poly(methyl methacrylate), sapphire, silicon, germanium, cyclic olefin copolymer, polyethylene, polypropylene, polyacrylate, polycarbonate, plastic, and combinations thereof.

In some cases the device comprises at least 1,000 partitions. In some cases the partitions have a density selected from the group consisting of at least about 1,000 partitions/cm^2 and at least about 10,000 partitions/cm^2.

In some cases the partitions are disposed along a fluid flow path having a fluid inlet and a fluid outlet.

In some cases the partitions are wells are disposed within a glass slide. In some cases the partitions are spots disposed on a glass slide.

In some cases the partitions have an interior surface comprising a hydrophilic material. In some cases a surface exterior to the partitions comprises a hydrophobic material. In some cases the fluid flow path comprises a surface comprising a hydrophobic material.

IV. Devices Comprising Spots in Wells

In some cases this disclosure provides devices comprising a plurality of wells, wherein at least one well of the plurality of wells comprises a plurality of discrete spots disposed on a surface within the well, wherein each spot comprises a species and the spots are configured to release the species upon application of a stimulus.

In some cases the device is formed from a material selected from the group consisting of fused silica, soda lime glass, borosilicate glass, poly(methyl methacrylate), sapphire, silicon, germanium, cyclic olefin copolymer, polyethylene, polypropylene, polyacrylate, polycarbonate, plastic, and combinations thereof.

In some cases the device comprises at least 1,000 wells. In some cases the wells have a density selected from the group consisting of at least about 1,000 wells/cm^2 and at least about 10,000 wells/cm^2.

In some cases the wells are disposed along a fluid flow path having a fluid inlet and a fluid outlet.

In some cases the wells are disposed within a glass slide. In some cases the spots are disposed on a glass slide.

In some cases the wells have an interior surface comprising a hydrophilic material. In some cases a surface exterior to the wells comprises a hydrophobic material. In some cases the fluid flow path comprises a surface comprising a hydrophobic material.

IV. Methods Utilizing Capsules in Droplets

In some cases this disclosure provides a method comprising: (a) providing a plurality of capsules, the capsules situated within droplets in an emulsion, wherein the capsules are configured to release their contents into the droplets upon the application of a stimulus; and (b) providing a stimulus to cause the capsules to release their contents into the droplets.

In some cases the capsules comprise a species selected from the group consisting of a reagent and an analyte. In some cases the droplets comprise a species selected from the group consisting of a reagent and an analyte.

In some cases causing the capsules to release their contents into the droplets results in contact between a polynucleotide and an enzyme. In some cases causing the capsules to release their contents into the droplets results in contact between a polynucleotide and a barcode.

In some cases the analyte is a polynucleotide present in a predetermined coverage amount.

In some cases the method further comprises the step of sequencing the polynucleotide. In some cases the method further comprises the step of performing a polynucleotide phasing analysis.

V. Methods Utilizing Capsules in Capsules

In some cases this disclosure provides a method comprising: (a) providing a plurality of inner capsules, the inner capsules situated within outer capsules, wherein the inner capsules are configured to release their contents into the outer capsules upon the application of a stimulus; and (b) providing a stimulus to cause the inner capsules to release their contents into the outer capsules.

In some cases the inner capsules comprise a species selected from the group consisting of a reagent and an analyte. In some cases the outer capsules comprise a species selected from the group consisting of a reagent and an analyte.

In some cases causing the inner capsules to release their contents into the outer capsules results in contact between a polynucleotide and an enzyme. In some cases causing the inner capsules to release their contents into the outer capsules results in contact between a polynucleotide and a barcode.

In some cases the analyte is a polynucleotide present in a predetermined coverage amount.

In some cases the method further comprises the step of sequencing the polynucleotide. In some cases the method further comprises the step of performing a polynucleotide phasing analysis.

VI. Methods Utilizing Spots in Wells

In some cases this disclosure provides a method comprising: (a) providing a well comprising a plurality of discrete spots disposed on a surface within the well, wherein each spot comprises a species and the spots are configured to release the species upon application of a stimulus; (b) adding a medium comprising a species to the well; and (c) providing a stimulus to cause the spots to release their contents into the well.

In some cases the spots comprise a species selected from the group consisting of a reagent and an analyte. In some cases the medium comprises a species selected from the group consisting of a reagent and an analyte.

In some cases causing the spots to release their contents into the well results in contact between a polynucleotide and an enzyme. In some cases causing the spots to release their contents into the well results in contact between a polynucleotide and a barcode.

In some cases the analyte is a polynucleotide present in a predetermined coverage amount.

In some cases the method further comprises the step of sequencing the polynucleotide. In some cases the method further comprises the step of performing a polynucleotide phasing analysis.

VI. Methods of Encapsulating Polynucleotides, Including Whole Chromosomes

In some cases this disclosure provides a method comprising: (a) providing a sample comprising a cell; (b) lysing the cell, thereby generating a lysate; and (c) partitioning the lysate into a capsule.

In some cases the sample comprises a plurality of cells. In some cases the plurality of cells comprises 1 to 100,000 cells, 10 to 10,000 cells, 100 to 5,000 cells, or 1,000 to 5,000 cells.

In some cases lysing comprises a treatment with a proteinase. In some cases the proteinase is proteinase K.

In some cases after the partitioning, at least one of the capsules comprises a single copy of a polynucleotide from the cell. In some cases, at least one capsule comprises a mixture of polynucleotides, wherein none of the polynucleotides in the mixture are overlapping.

In some cases the polynucleotide is a chromosome.

In some cases the capsule comprises a shell with pores that restrict trans-shell transport of the polynucleotide but allow trans-shell transport of a species. In some cases the method further comprises the step of transporting an inner species from the interior of the capsule to the exterior of the capsule. In some cases the inner species is selected from the group consisting of a component of a buffer, a component of a cell, and a macromolecule. In some cases the method further comprises the step of transporting an outer species from the exterior of the capsule to the interior of the capsule. In some cases the outer species is selected from the group consisting of a reagent, a protein, a polynucleotide, an enzyme, an antibody, a barcode, an adapter, a buffer, a small molecule, a detergent, a dye, a polymer and combinations thereof. In some cases the outer species is a reagent for nucleic acid amplification.

In some cases the method further comprises the step of amplifying the encapsulated polynucleotide. In some cases the amplifying is performed by a method selected from the group consisting of multiple displacement amplification, polymerase chain reaction, ligase chain reaction, helicase-dependent amplification, and combinations thereof.

In some cases the method further comprises the step of fragmenting the encapsulated polynucleotide, thereby generating a fragmented polynucleotide. In some cases the method further comprises the step of attaching a barcode to the fragmented polynucleotide.

In some cases the method further comprises the step of placing the capsule comprising polynucleotide (or processed polynucleotide) into a partition. In some cases the partition is selected from the group consisting of a well, a droplet in an emulsion, and a capsule.

In some cases the capsule comprising the polynucleotide is configured to release its contents upon the application of a stimulus. In some cases the stimulus is selected from the group consisting of a chemical stimulus, a bulk stimulus, a biological stimulus, a light stimulus, a thermal stimulus, a magnetic stimulus, and combinations thereof.

In some cases the method further comprises the step of performing a polynucleotide phasing analysis.

VII. Kits

In some cases this disclosure provides kits. In some cases the disclosure provides kits for generating capsules within droplets in an emulsion, the kits comprising reagents for generating capsules, reagents for generating an emulsion, and instructions for generating the capsules within droplets in an emulsion. In some cases the capsules are configured to release their contents into the droplets upon the application of a stimulus. In some cases the kits further comprise a species for inclusion in a partition selected from the group consisting of the capsules, the droplets, and combinations thereof.

In some cases, this disclosure provides kits for generating capsules within capsules, the kits comprising reagents for generating inner capsules, reagents for generating outer capsules, and instructions for generating capsules within capsules. In some cases the inner capsules are configured to release their contents into the outer capsules upon the application of a stimulus. In some cases, the kits further comprise a species for inclusion in a partition selected from the group consisting of the inner capsules, the outer capsules, and combinations thereof.

In some cases this disclosure provides kits comprising a plurality of discrete spots disposed on a surface within a well, wherein each spot comprises a species and the spots are configured to release the species upon application of a stimulus, and instructions for use of the kit to process a sample. In some cases the stimulus is the addition of a sample to the well.

VIII. Partitioning and Fragmenting Methods

In some cases this disclosure provides a method of partitioning polynucleotides comprising: (a) isolating polynucleotides from a source of polynucleotides; (b) partitioning the polynucleotides at a predetermined coverage amount, to produce a plurality of partitions, wherein at least one partition comprises a mixture of non-overlapping polynucleotides, thereby generating partitioned polynucleotides; and (c) fragmenting the partitioned polynucleotides, thereby generating fragmented polynucleotides.

In some cases the source is a cell.

In some cases at least about 50% of the partitions comprise a mixture of non-overlapping polynucleotides.

In some cases the method further comprises the step of amplifying the partitioned polynucleotides. In some cases, the method further comprises the step of barcoding the fragmented polynucleotides.

In some cases this disclosure provides a method of fragmenting a polynucleotide comprising: (a) providing a polynucleotide; (b) encapsulating the polynucleotide, thereby generating an encapsulated polynucleotide; and (c) fragmenting the polynucleotide, thereby generating an encapsulated fragmented polynucleotide.

In some cases the fragmenting is performed by ultrasonic waves.

In some cases the encapsulating disposes the polynucleotide within a capsule. In some cases the encapsulating disposes the polynucleotide within a droplet of a hydrogel.

In some cases the method further comprises the step of encapsulating the encapsulated polynucleotide within a capsule. In some cases the method further comprises the step of encapsulating the encapsulated polynucleotide within a droplet of a hydrogel.

An additional aspect of the disclosure provides the use of a composition, device, method, or kit described herein in partitioning species, in partitioning oligonucleotides, in stimulus-selective release of species from partitions, in performing reactions (e.g., ligation and amplification reactions) in partitions, in performing nucleic acid synthesis reactions, in barcoding nucleic acid, in preparing polynucleotides for sequencing, in sequencing polynucleotides, in mutation detection, in neurologic disorder diagnostics, in diabetes diagnostics, in fetal aneuploidy diagnostics, in

cancer mutation detection and forensics, in disease detection, in medical diagnostics, in low input nucleic acid applications, in circulating tumor cell (CTC) sequencing, in polynucleotide phasing, in sequencing polynucleotides from small numbers of cells, in analyzing gene expression, in partitioning polynucleotides from cells, or in a combination thereof.

INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

The novel features of methods, compositions, systems, and devices of this disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of this disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the methods, compositions, systems, and devices of this disclosure are utilized, and the accompanying drawings of which:

FIGS. **1**A-B are schematic examples of wells comprising other types of partitions.

FIGS. **2**A-B are schematic examples of spots comprising other types of partitions.

FIGS. **3**A-B are schematic examples of a droplet of an emulsion comprising other types of partitions.

FIGS. **4**A-B are schematic examples of capsules comprising other types of partitions.

FIG. **5** is a schematic example of a flow focusing method for the production of capsules and/or emulsions.

FIG. **6** is a schematic example of a flow focusing method for the production of capsules within capsules, capsules within droplets of an emulsion, or droplets of an emulsion within capsules.

FIG. **7** is a schematic example of a method for the batch production of capsules.

FIGS. **8**A-**8**D are schematic examples of partition-in-partition configurations.

FIGS. **9**A-B provide micrographs of capsules formed as described in Example 1.

FIG. **10** provides a micrograph of a capsule formed as described in Example 2.

FIG. **11** provides micrographs of temperature-responsive capsules formed as described in Example 3.

FIG. **12** provides micrographs of chemically-responsive capsules formed as described in Example 4.

FIGS. **13**A-E provide schematic examples of certain configurations of partitions, as described in Example 5.

FIG. **14** provides a schematic example of discrete spots on a surface of a well, as described in Example 6.

FIG. **15**A-B provide schematic examples of shearing encapsulated DNA and exposing it to reagents for further processing, as described in Example 7.

DETAILED DESCRIPTION

While various embodiments of the invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions may occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed.

This disclosure provides methods, compositions, systems, devices, and kits for partitioning and processing of species. A species may be contained within a sample that may also comprise other species. The sample may be partitioned. A sample may comprise any suitable species, as described more fully elsewhere in this disclosure. In some cases, a sample comprises a species that is a reagent or an analyte. The methods, compositions, systems, devices, and kits may be used for a variety of applications. Analyte may be processed for any suitable application, including, for example, processing polynucleotides for polynucleotide sequencing. Polynucleotides sequencing includes the sequencing of whole genomes, detection of specific sequences such as single nucleotide polymorphisms (SNPs) and other mutations, detection of nucleic acid (e.g., deoxyribonucleic acid) insertions, and detection of nucleic acid deletions.

Utilization of the methods, compositions, systems, devices, and kits described herein may incorporate, unless otherwise indicated, any conventional techniques of organic chemistry, polymer technology, microfluidics, molecular biology, recombinant techniques, cell biology, biochemistry, and immunology. Such conventional techniques include well and microwell construction, capsule generation, generation of emulsions, spotting, microfluidic device construction, polymer chemistry, restriction digestion, ligation, cloning, polynucleotide sequencing, and polynucleotide sequence assembly. Specific, non-limiting, illustrations of suitable techniques are described throughout this disclosure. However, equivalent procedures may also be utilized. Descriptions of certain techniques may be found in standard laboratory manuals, such as *Genome Analysis: A Laboratory Manual Series* (*Vols. I-IV*), *Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), and "Oligonucleotide Synthesis: A Practical Approach" 1984, IRL Press London, all of which are herein incorporated in their entirety by reference for all purposes.

I. DEFINITIONS

The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including," "includes," "having," "has," "with," "such as," or variants thereof, are used in either the specification and/or the claims, such terms are not limiting and are intended to be inclusive in a manner similar to the term "comprising".

The term "about," as used herein, generally refers to a range that is 15% greater than or less than a stated numerical value within the context of the particular usage. For example, "about 10" would include a range from 8.5 to 11.5.

The term "barcode," as used herein, generally refers to a label that may be attached to an analyte to convey information about the analyte. For example, a barcode may be a polynucleotide sequence attached to all fragments of a target polynucleotide contained within a particular partition. This barcode may then be sequenced with the fragments of the target polynucleotide. The presence of the same barcode on

multiple sequences may provide information about the origin of the sequence. For example, a barcode may indicate that the sequence came from a particular partition and/or a proximal region of a genome. This may be particularly useful when several partitions are pooled before sequencing.

The term "bp," as used herein, generally refers to an abbreviation for "base pairs".

The term "microwell," as used herein, generally refers to a well with a volume of less than 1 mL. Microwells may be made in various volumes, depending on the application. For example, microwells may be made in a size appropriate to accommodate any of the partition volumes described herein.

The term "partition," as used herein, may be a verb or a noun. When used as a verb (e.g., "to partition," or "partitioning"), the term generally refers to the fractionation (subdivision) of a species or sample (e.g., a polynucleotide) between vessels that can be used to sequester one fraction (or subdivision) from another. Such vessels are referred to using the noun "partition." Partitioning may be performed, for example, using microfluidics, dilution, dispensing, and the like. A partition may be, for example, a well, a microwell, a hole, a droplet (e.g., a droplet in an emulsion), a continuous phase of an emulsion, a test tube, a spot, a capsule, or any other suitable container for sequestering one fraction of a sample from another.

The terms "polynucleotide" or "nucleic acid," as used herein, generally refer to molecules comprising a plurality of nucleotides. Exemplary polynucleotides include deoxyribonucleic acids, ribonucleic acids, and synthetic analogues thereof, including peptide nucleic acids.

The term "species," as used herein, generally refers to any substance that can be used with the methods, compositions, systems, devices, and kits of this disclosure. Examples of species include reagents, analytes, cells, chromosomes, tagging molecules or groups of molecules, barcodes, and any sample comprising any of these species. Any suitable species may be used, as more fully discussed elsewhere in this disclosure.

II. PARTITIONS

a. General Characteristics of Partitions

As described throughout this disclosure, certain methods, compositions, systems, devices, and kits of the disclosure may utilize the subdivision (partitioning) of certain species into separate partitions. A partition may be, for example, a well, a microwell, a hole, a droplet (e.g., a droplet in an emulsion), a continuous phase of an emulsion, a test tube, a spot, a bead (e.g., a gel bead, a paraffin bead, a wax bead), a capsule, or any other suitable container for sequestering one fraction of a sample or a species. In some cases, a capsule is a bead (e.g., a gel bead). Partitions may be used to contain a species for further processing. For example, if a species is a polynucleotide analyte, further processing may comprise cutting, ligating, and/or barcoding with species that are reagents. Any number of devices, systems or containers may be used to hold, support or contain partitions. In some cases, a microwell plate may be used to hold, support, or contain partitions. Any suitable microwell plate may be used, for example microwell plates having 96, 384, or 1536 wells.

Each partition may also contain, or be contained within any other suitable partition. For example, a well, microwell, hole, or tube may comprise a droplet (e.g., a droplet in an emulsion), a continuous phase in an emulsion, a spot, a capsule, or any other suitable partition. A droplet may comprise a capsule or another droplet. A capsule may comprise a droplet or another capsule. These descriptions are merely illustrative, and all suitable combinations and pluralities are also envisioned. For example, any suitable partition may comprise a plurality of the same or different partitions. In one example, a well or microwell comprises a plurality of droplets and a plurality of capsules. In another example, a capsule comprises a plurality of capsules and a plurality of droplets. All combinations of partitions are envisioned. Table 1 shows non-limiting examples of partitions that may be combined with each other.

TABLE 1

Examples of partitions that may be combined with each other.

	Well	Spot	Droplet	Capsule
Well	Well inside well	Spot inside well	Droplet inside well	Capsule inside well
Spot	Spot inside well	Spot inside spot	Droplet inside spot	Capsule inside spot
Droplet	Droplet inside well	Droplet inside spot	Droplet inside droplet	Droplet inside capsule Capsule inside droplet
Capsule	Capsule inside well	Capsule inside spot Spot inside capsule	Capsule inside droplet Droplet inside capsule	Capsule inside capsule

Any partition described herein may comprise multiple partitions. For example, a partition may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 partitions. A partition may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 partitions. In some cases, a partition may comprise less than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 partitions. In some cases, each partition may comprise 2-50, 2-20, 2-10, or 2-5 partitions.

A partition may comprise any suitable species or mixture of species. For example, in some cases a partition may comprise a reagent, an analyte, a sample, a cell, and combinations thereof. A partition comprising other partitions may comprise certain species in the same partitions and certain species in different partitions. Species may be distributed between any suitable partitions, depending on the needs of the particular process. For example, any of the partitions in Table 1 may contain at least one first species and any of the partitions in Table 1 may contain at least one second species. In some cases the first species may be a reagent and the second species may be an analyte.

In some cases, a species is a polynucleotide isolated from a cell. For example, in some cases polynucleotides (e.g., genomic DNA, RNA, etc.) is isolated from a cell utilizing any suitable method (e.g., a commercially available kit). The polynucleotide may be quantified. The quantified polynucleotide may then be partitioned into a plurality of partitions as described herein. The partitioning of the polynucleotide may be performed at a predetermined coverage amount, according to the quantification and the needs of the assay. In some cases, all or most partitions do not comprise polynucleotides that overlap, such that separate mixtures of non-overlapping fragments are formed across the plurality of partitions. The

partitioned polynucleotides may then be treated according to any suitable method known in the art or described in this disclosure. For example, the partitioned polynucleotides may be fragmented, amplified, barcoded, and the like.

Species may be partitioned using a variety of methods. For example, species may be diluted and dispensed across a plurality of partitions. A terminal dilution of a medium comprising species may be performed such that the number of partitions exceeds the number of species. Dilution may also be used prior to forming an emulsion or capsules, or prior to spotting a species on a substrate. The ratio of the number of species to the number of partitions may be about 0.1, 0.5, 1, 2, 4, 8, 10, 20, 50, 100, or 1000. The ratio of the number of species to the number of partitions may be at least about 0.1, 0.5, 1, 2, 4, 8, 10, 20, 50, 100, or 1000. The ratio of the number of species to the number of partitions may be less than about 0.1, 0.5, 1, 2, 4, 8, 10, 20, 50, 100, or 1000. The ratio of the number of species to the number of partitions may range from about 0.1-10, 0.5-10, 1-10, 2-10, 10-100, 100-1000, or more.

Partitioning may also be performed using piezoelectric droplet generation (e.g., Bransky et al., *Lab on a Chip,* 2009, 9, 516-520) or surface acoustic waves (e.g., Demirci and Montesano, *Lab on a Chip,* 2007, 7, 1139-1145).

The number of partitions employed may vary depending on the application. For example, the number of partitions may be about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, or 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100,000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1,000,000, 2000000, 3000000, 4000000, 5000000, 10000000, 20000000 or more. The number of partitions may be at least about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1000000, 2000000, 3000000, 4000000, 5000000, 10000000, 20000000 or more. The number of partitions may be less than about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1000000, 2000000, 3000000, 4000000, 5000000, 10000000, or 20000000. The number of partitions may be about 5-10000000, 5-5000000, 5-1,000,000, 10-10,000, 10-5,000, 10-1,000, 1,000-6,000, 1,000-5,000, 1,000-4,000, 1,000-3,000, or 1,000-2,000.

The volume of the partitions may vary depending on the application. For example, the volume of any of the partitions described in this disclosure (e.g., wells, spots, droplets (e.g., in an emulsion), and capsules) may be about 1000 μl, 900 μl, 800 μl, 700 μl, 600 μl, 500 μl, 400 μl, 300 μl, 200 μl, 100 μl, 50 μl, 25 μl, 10 μl, 5 μl, 1 μl, 900 nL, 800 nL, 700 nL, 600 nL, 500 nL, 400 nL, 300 nL, 200 nL, 100 nL, 50 nL, 25 nL, 10 nL, 5 nL, 2.5 nL, 1 nL, 900 pL, 800 pL, 700 pL, 600 pL, 500 pL, 400 pL, 300 pL, 200 pL, 100 pL, 50 pL, 25 pL, 10 pL, 5 pL, 1 pL, 900 fL, 800 fL, 700 fL, 600 fL, 500 fL, 400 fL, 300 fL, 200 fL, 100 fL, 50 fL, 25 fL, 10 fL, 5 fL, 1 fL, or 0.5 fL. The volume of the partitions may be at least about 1000 μl, 900 μl, 800 μl, 700 μl, 600 μl, 500 μl, 400 μl, 300 μl, 200 μl, 100 μl, 50 μl, 25 μl, 10 μl, 5 μl, 1 μl, 900 nL, 800 nL, 700 nL, 600 nL, 500 nL, 400 nL, 300 nL, 200 nL, 100 nL, 50 nL, 25 nL, 10 nL, 5 nL, 5 nL, 2.5 nL, 1 nL, 900 pL, 800 pL, 700 pL, 600 pL, 500 pL, 400 pL, 300 pL, 200 pL, 100 pL, 50 pL, 25 pL, 10 pL, 5 pL, 1 pL, 900 fL, 800 fL, 700 fL, 600 fL, 500 fL, 400 fL, 300 fL, 200 fL, 100 fL, 50 fL, 25 fL, 10 fL, 5 fL, 1 fL, or 0.5 fL. The volume of the partitions may be less than about 1000 μl, 900 μl, 800 μl, 700 μl, 600 μl, 500 μl, 400 μl, 300 μl, 200 μl, 100 μl, 50 μl, 25 μl, 10 μl, 5 μl, 1 μl, 900 nL, 800 nL, 700 nL, 600 nL, 500 nL, 400 nL, 300 nL, 200 nL, 100 nL, 50 nL, 25 nL, 10 nL, 5 nL, 5 nL, 2.5 nL, 1 nL, 900 pL, 800 pL, 700 pL, 600 pL, 500 pL, 400 pL, 300 pL, 200 pL, 100 pL, 50 pL, 25 pL, 10 pL, 5 pL, 1 pL, 900 fL, 800 fL, 700 fL, 600 fL, 500 fL, 400 fL, 300 fL, 200 fL, 100 fL, 50 fL, 25 fL, 10 fL, 5 fL, 1 fL, or 0.5 fL. the volume of the partitions may be about 0.5 fL-5 pL, 10 pL-10 nL, 10 nL-10 μl, 10 μl-100 μl, or 100 μl to 1 mL.

There may be variability in the volume of fluid in different partitions. More specifically, the volume of different partitions may vary by at least (or at most) plus or minus 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, or 1000% across a set of partitions. For example, a well (or other partition) may comprise a volume of fluid that is at most 80% of the fluid volume within a second well (or other partition).

Particular species may also be targeted to specific partitions. For example, in some cases, a capture reagent (e.g., an oligonucleotide probe) may be immobilized or placed within a partition to capture specific species (e.g., polynucleotides).

The number of different species or different sets of species that are partitioned may vary depending upon, for example, the particular species to be partitioned and/or the application. Different sets of species may be, for example, sets of identical species where the identical species differ between each set. Or different sets of species may be, for example, sets of different species, where each set differs in its included species. For example, about 1, 5, 10, 50, 100, 1000, 10000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, 100000000, or more different species or different sets of species may be partitioned. In some examples, at least about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, 100000000, or more different species or different sets of species may be partitioned. In some examples, less than about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 different species or different sets of species may be partitioned. In some examples, about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, 100000-1000000, 10000-1000000, 10000-10000000, or 10000-100000000 species may be partitioned.

Species may also be partitioned at a particular density. For example, species may be partitioned so that each partition contains about 1, 5, 10, 50, 100, 1000, 10000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, or 20000000 species per partition. Species may be partitioned so that each partition contains at least about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000,

400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, or 20000000 or more species per partition. Species may be partitioned so that each partition contains less than about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, or 20000000 species per partition. Species may be partitioned such that each partition contains about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, 100000-1000000, 10000-1000000, or 10000-10000000 species per partition.

Species may also be partitioned such that identical species are partitioned at a particular density. For example, identical species may be partitioned so that each partition contains about 1, 5, 10, 50, 100, 1000, 10000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, or 20000000 identical species per partition. Species may be partitioned so that each partition contains at least about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, or 20000000 or more identical species per partition. Species may be partitioned so that each partition contains less than about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, or 20000000 identical species per partition. Species may be partitioned such that each partition contains about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, 100000-1000000, 10000-1000000, or 10000-10000000 identical species per partition.

Species may also be partitioned such that different species are partitioned at a particular density. For example, different species may be partitioned so that each partition contains about 1, 5, 10, 50, 100, 1000, 10000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, or 20000000 different species per partition. Species may be partitioned so that each partition contains at least about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, or 20000000 or more different species per partition. Species may be partitioned so that each partition contains less than about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, or 20000000 different species per partition. Species may be partitioned such that each partition contains about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, 100000-1000000, 10000-1000000, or 10000-10000000 different species per partition.

As will be appreciated, any of the above-described different numbers of species may be provided with any of the above-described barcode densities per partition, and in any of the above-described numbers of partitions.

Species may be partitioned such that at least one partition comprises a species that is unique within that partition. This may be true for about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of the partitions. This may be true for at least about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of the partitions. This may be true for less than about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the partitions.

a. Wells as Partitions

In some cases, wells are used as partitions. The wells may be microwells. With reference to FIG. **1**A, a well **101** may comprise a medium **102** comprising a species or plurality of species **103**. Species may be contained within a well in various configurations. In one example, a species is dispensed directly into a well (e.g., FIG. **1**A). A species dispensed directly into a well may be overlaid with a layer that is, for example, dissolvable, meltable, or permeable **104**. This layer may be, for example, an oil, wax, membrane, or the like. The layer may be dissolved or melted prior to or after introduction of another species into the well. The well may be sealed at any point, with a sealing layer **105**, for example after addition of any species.

In one example, reagents for sample processing are dispensed directly into a well and overlaid with a layer that is dissolvable, meltable, or permeable. A sample comprising an analyte to be processed is introduced on top of the layer. The layer is dissolved or melted, or the analyte (or reagent) diffuses through the layer. The well is sealed and incubated under appropriate conditions for the processing of the analyte. Processed analyte may then be recovered.

In some cases, wells comprise other partitions. A well may comprise any suitable partition including, for example, with reference to FIG. **1**B, another well **106**, a spot **107**, a droplet (e.g., a droplet in an emulsion) **108**, a capsule **109**, and the like. Each partition may be present as a single partition or a plurality of partitions, and each partition may comprise the same species or different species.

In one example, a well comprises a capsule comprising reagents for sample processing. A capsule may be loaded into a well using a liquid medium, or loaded into a well without a liquid medium (e.g., essentially dry). As described elsewhere in this disclosure, a capsule may contain one or more capsules, or other partitions. A sample comprising an analyte to be processed is introduced into the well. The well is sealed and a stimulus is applied to cause release of the contents of the capsule into the well, resulting in contact between the reagents and the analyte to be processed. The well is incubated under appropriate conditions for the processing of the analyte. Processed analyte may then be recovered. While this example describes an embodiment where a reagent is in a capsule and an analyte is in the well, the opposite configuration—i.e., reagent in the well and analyte in the capsule—is also possible.

In another example, a well comprises an emulsion and the droplets of the emulsion comprise capsules comprising reagents for sample processing. A sample comprising an analyte to be processed is contained within the droplets of the emulsion. The well is sealed and a stimulus is applied to cause release of the contents of the capsules into the droplets, resulting in contact between the reagents and the analyte to be processed. The well is incubated under appropriate conditions for the processing of the analyte. Processed analyte may then be recovered. While this example

describes an embodiment where a reagent is in a capsule and an analyte is in a droplet, the opposite configuration—i.e., reagent in the droplet and analyte in the capsule—is also possible.

Wells may be arranged as an array, for example a microwell array. Based on the dimensions of individual wells and the size of the substrate, the well array may comprise a range of well densities. In some cases, the well density may be 10 wells/cm^2, 50 wells/cm^2, 100 wells/cm^2, 500 wells/cm^2, 1000 wells/cm^2, 5000 wells/cm^2, 10000 wells/cm^2, 50000 wells/cm^2, or 100000 wells/cm^2. In some cases, the well density may be at least 10 wells/cm^2, 50 wells/cm^2, 100 wells/cm^2, 500 wells/cm^2, 1000 wells/cm^2, 5000 wells/cm^2, 10000 wells/cm^2, 50000 wells/cm^2, or 100000 wells/cm^2. In some cases, the well density may be less than 10 wells/cm^2, 50 wells/cm^2, 100 wells/cm^2, 500 wells/cm^2, 1000 wells/cm^2, 5000 wells/cm^2, 10000 wells/cm^2, 50000 wells/cm^2, or 100000 wells/cm^2.

b. Spots as Partitions

In some cases, spots are used as partitions. With reference to FIG. **2**A, a spot may be made, for example, by dispensing a substance **201** on a surface **202**. Species may be contained within a spot in various configurations. In one example, a species is dispensed directly into a spot by including the species in the medium used to form the spot. A species dispensed directly onto a spot may be overlaid with a layer that is, for example, dissolvable, meltable, or permeable **203**. This layer may be, for example, an oil, wax, membrane, or the like. The layer may be dissolved or melted prior to or after introduction of another species onto the spot. The spot may be sealed at any point, for example after addition of any species, by an overlay **204**.

In one example, reagents for sample processing are dispensed directly onto a spot, for example on a glass slide, and overlaid with a layer that is dissolvable, meltable, or permeable. A sample comprising an analyte to be processed is introduced on top of the layer. The layer is dissolved or melted, or the analyte (or reagent) diffuses through the layer. The spot is sealed and incubated under appropriate conditions for the processing of the analyte. Processed analyte may then be recovered.

As described elsewhere in this disclosure (e.g., Table 1), spots may also be arranged within a well. In some cases, a plurality of spots may be arranged within a well such that the contents of each spot do not mix. Such a configuration may be useful, for example, when it is desirable to prevent species from contacting each other. In some cases, a well may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more spots. In some cases, a well may comprise at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more spots. In some cases, a well may comprise less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 spots. In some cases, a well may comprise 2-4, 2-6, 2-8, 4-6, 4-8, 5-10, or 4-12 spots. Upon addition of a substance (e.g., a medium containing an analyte) to the well, the species in the spot may mix. Moreover, using separate spots to contain different species (or combinations of species) may also be useful to prevent cross-contamination of devices used to place the spots inside the well.

In some cases, spots comprise other partitions. A spot may comprise any suitable partition including, for example, with reference to FIG. **2**B, another spot **205**, a droplet (e.g., a droplet in an emulsion) **206**, a capsule **207**, and the like. Each partition may be present as a single partition or a plurality of partitions, and each partition may comprise the same species or different species.

In one example, a spot comprises a capsule comprising reagents for sample processing. As described elsewhere in this disclosure, a capsule may contain one or more capsules, or other partitions. A sample comprising an analyte to be processed is introduced into the spot. The spot is sealed and a stimulus is applied to cause release of the contents of the capsule into the spot, resulting in contact between the reagents and the analyte to be processed. The spot is incubated under appropriate conditions for the processing of the analyte. Processed analyte may then be recovered. While this example describes an embodiment where a reagent is in a capsule and an analyte is in the spot, the opposite configuration—i.e., reagent in the spot and analyte in the capsule—is also possible.

In another example, a spot comprises an emulsion and the droplets of the emulsion comprise capsules comprising reagents for sample processing. A sample comprising an analyte to be processed is contained within the droplets of the emulsion. The spot is sealed and a stimulus is applied to cause release of the contents of the capsules into the droplets, resulting in contact between the reagents and the analyte to be processed. The spot is incubated under appropriate conditions for the processing of the analyte. Processed analyte may then be recovered. While this example describes an embodiment where a reagent is in a capsule and an analyte is in a droplet, the opposite configuration—i.e., reagent in the droplet and analyte in the capsule—is also possible.

Spots may be of uniform size or heterogeneous size. In some cases, the diameter of a spot may be about 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 150 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, 1 mm, 2 mm, 5 mm, or 1 cm. A spot may have a diameter of at least about 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 150 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, 1 mm, 1 mm, 2 mm, 5 mm, or 1 cm. In some cases, a spot may have a diameter of less than about 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 150 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, 1 mm, 1 mm, 2 mm, 5 mm, or 1 cm. In some cases, a spot may have a diameter of about 0.1 μm to 1 cm, 100 μm to 1 mm, 100 μm to 500 μm, 100 μm to 600 μm, 150 μm to 300 μm, or 150 μm to 400 μm.

Spots may be arranged as an array, for example a spot array. Based on the dimensions of individual spots and the size of the substrate, the spot array may comprise a range of spot densities. In some cases, the spot density may be 10 spots/cm^2, 50 spots/cm^2, 100 spots/cm^2, 500 spots/cm^2, 1000 spots/cm^2, 5000 spots/cm^2, 10000 spots/cm^2, 50000 spots/cm^2, or 100000 spots/cm^2. In some cases, the spot density may be at least 10 spots/cm^2, 50 spots/cm^2, 100 spots/cm^2, 500 spots/cm^2, 1000 spots/cm^2, 5000 spots/cm^2, 10000 spots/cm^2, 50000 spots/cm^2, or 100000 spots/cm^2. In some cases, the spot density may be less than 10 spots/cm^2, 50 spots/cm^2, 100 spots/cm^2, 500 spots/cm^2, 1000 spots/cm^2, 5000 spots/cm^2, 10000 spots/cm^2, 50000 spots/cm^2, or 100000 spots/cm^2.

c. Emulsions as Partitions

In some cases, the droplets in an emulsion are used as partitions. An emulsion may be prepared, for example, by any suitable method, including methods known in the art. (See e.g., Weizmann et al., Nature Methods, 2006, 3(7):545-550; Weitz et al. U.S. Pub. No. 2012/0211084). In some cases, water-in-fluorocarbon emulsions may be used. These emulsions may incorporate fluorosurfactants such as oligo-

meric perfluorinated polyethers (PFPE) with polyethylene glycol (PEG). (Holtze et al., Lab on a Chip, 2008, 8(10): 1632-1639). In some cases, monodisperse emulsions may be formed in a microfluidic flow focusing device. (Garstecki et al., Applied Physics Letters, 2004, 85(13):2649-2651).

FIG. **3**A illustrates exemplary embodiments. With reference to FIG. **3**A, a species may be contained within a droplet **301** in an emulsion containing, for example, a first phase (e.g., oil or water) forming the droplet **301** and a second (continuous) phase (e.g., water or oil) **302**. An emulsion may be a single emulsion, for example, a water-in-oil or an oil-in-water emulsion. An emulsion may be a double emulsion, for example a water-in-oil-in-water or an oil-in-water-in-oil emulsion. Higher-order emulsions are also possible. The emulsion may be held in any suitable container, including any suitable partition described in this disclosure.

In some cases, droplets in an emulsion comprise other partitions. A droplet in an emulsion may comprise any suitable partition including, for example, with reference to FIG. **3**B, another droplet (e.g., a droplet in an emulsion) **303**, a capsule **304**, and the like. Each partition may be present as a single partition or a plurality of partitions, and each partition may comprise the same species or different species.

In one example, a droplet in an emulsion comprises a capsule comprising reagents for sample processing. As described elsewhere in this disclosure, a capsule may contain one or more capsules, or other partitions. A sample comprising an analyte to be processed is contained within the droplet. A stimulus is applied to cause release of the contents of the capsule into the droplet, resulting in contact between the reagents and the analyte to be processed. The droplet is incubated under appropriate conditions for the processing of the analyte. Processed analyte may then be recovered. While this example describes an embodiment where a reagent is in a capsule and an analyte is in the droplet, the opposite configuration—i.e., reagent in the droplet and analyte in the capsule—is also possible.

The droplets in an emulsion may be of uniform size or heterogeneous size. In some cases, the diameter of a droplet in an emulsion may be about 0.001 μm, 0.01 μm, 0.05 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 150 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, or 1 mm. A droplet may have a diameter of at least about 0.001 μm, 0.01 μm, 0.05 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 150 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, or 1 mm. In some cases, a droplet may have a diameter of less than about 0.001 μm, 0.01 μm, 0.05 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 150 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, or 1 mm. In some cases, a droplet may have a diameter of about 0.001 μm to 1 mm, 0.01 μm to 900 μm, 0.1 μm to 600 μm, 100 μm to 200 μm, 100 μm to 300 μm, 100 μm to 400 μm, 100 μm to 500 μm, 100 μm to 600 μm, 150 μm to 200 μm, 150 μm to 300 μm, or 150 μm to 400 μm.

Droplets in an emulsion also may have a particular density. In some cases, the droplets are less dense than an aqueous fluid (e.g., water); in some cases, the droplets are denser than an aqueous fluid. In some cases, the droplets are less dense than a non-aqueous fluid (e.g., oil); in some cases, the droplets are denser than a non-aqueous fluid. Droplets may have a density of about 0.05 g/cm^3, 0.1 g/cm^3, 0.2 g/cm^3, 0.3 g/cm^3, 0.4 g/cm^3, 0.5 g/cm^3, 0.6 g/cm^3, 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. Droplets may have a density of at least about 0.05 g/cm^3, 0.1 g/cm^3, 0.2 g/cm^3, 0.3 g/cm^3, 0.4 g/cm^3, 0.5 g/cm^3, 0.6 g/cm^3, 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. In other cases, droplet densities may be at most about 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. Such densities can reflect the density of the capsule in any particular fluid (e.g., aqueous, water, oil, etc.)

d. Capsules as Partitions

In some cases, capsules are used as partitions. With reference to FIG. **4**A, a capsule **401** may be prepared by any suitable method, including methods known in the art, including emulsification polymerization (Weitz et al. (U.S. Pub. No. 2012/0211084)), layer-by-layer assembly with polyelectrolytes, coacervation, internal phase separation, and flow focusing. Any suitable species may be contained within a capsule. The capsule may be held in any suitable container, including any suitable partition described in this disclosure.

In some cases, capsules comprise other partitions. A capsule may comprise any suitable partition including, for example, with reference to FIG. **4**B, another capsule **402**, a droplet in an emulsion **403**, and the like. Each partition may be present as a single partition or a plurality of partitions, and each partition may comprise the same species or different species.

In one example, an outer capsule comprises an inner capsule. The inner capsule comprises reagents for sample processing. An analyte is encapsulated in the medium between the inner capsule and the outer capsule. A stimulus is applied to cause release of the contents of the inner capsule into the outer capsule, resulting in contact between the reagents and the analyte to be processed. The outer capsule is incubated under appropriate conditions for the processing of the analyte. Processed analyte may then be recovered. While this example describes an embodiment where a reagent is in an inner capsule and an analyte in the medium between the inner capsule and the outer capsule, the opposite configuration—i.e., reagent in the medium between the inner capsule and the outer capsule, and analyte in the inner capsule—is also possible.

Capsules may be pre-formed and filled with reagents by injection. For example, the picoinjection methods described in Abate et al. (Proc. Natl. Acad. Sci. U.S.A., 2010, 107(45), 19163-19166) and Weitz et al. (U.S. Pub. No. 2012/0132288) may be used to introduce reagents into the interior of capsules described herein. Generally, the picoinjection will be performed prior to the hardening of the capsule shell, for example by injecting species into the interior of a capsule precursor, such as a droplet of an emulsion, before formation of the capsule shell.

Capsules may be of uniform size or heterogeneous size. In some cases, the diameter of a capsule may be about 0.001 μm, 0.01 μm, 0.05 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 150 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, or 1 mm. A capsule may have a diameter of at least about 0.001 μm, 0.01 μm, 0.05 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 150 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, or 1 mm. In some cases, a capsule may have a diameter of less than about 0.001 μm, 0.01 μm, 0.05 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 150 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, or 1 mm. In some cases, a capsule may have a diameter of about 0.001 μm to 1 mm, 0.01 μm to 900 μm, 0.1 μm to 600 μm, 100 μm to 200 μm, 100 μm to 300 μm, 100 μm to 400 μm, 100 μm to 500 μm, 100 μm to 600 μm, 150 μm to 200 μm, 150 μm to 300 μm, or 150 μm to 400 μm.

Capsules also may have a particular density. In some cases, the capsules are less dense than an aqueous fluid (e.g., water); in some cases, the capsules are denser than an aqueous fluid. In some cases, the capsules are less dense than a non-aqueous fluid (e.g., oil); in some cases, the capsules are denser than a non-aqueous fluid. Capsules may have a density of about 0.05 g/cm^3, 0.1 g/cm^3, 0.2 g/cm^3, 0.3 g/cm^3, 0.4 g/cm^3, 0.5 g/cm^3, 0.6 g/cm^3, 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. Capsules may have a density of at least about 0.05 g/cm^3, 0.1 g/cm^3, 0.2 g/cm^3, 0.3 g/cm^3, 0.4 g/cm^3, 0.5 g/cm^3, 0.6 g/cm^3, 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. In other cases, capsule densities may be at most about 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. Such densities can reflect the density of the capsule in any particular fluid (e.g., aqueous, water, oil, etc.)

1. Production of Capsules by Flow Focusing

In some cases, capsules may be produced by flow focusing. Flow focusing is a method whereby a first fluid that is immiscible with a second fluid is flowed into the second fluid. With reference to FIG. **5**, a first (e.g., aqueous) fluid comprising a monomer, crosslinker, initiator, and aqueous surfactant **501** is flowed into a second (e.g., oil) fluid comprising a surfactant and an accelerator **502**. After entering the second fluid at a T-junction in a microfluidic device **503**, a droplet of first fluid breaks off from the first fluid stream and a capsule shell begins to form **504** due to the mixing of the monomer, crosslinker, and initiator in the first fluid and the accelerator in the second fluid. Thus, a capsule is formed. As the capsule proceeds downstream, the shell becomes thicker due to increased exposure to the accelerator. Varying the concentrations of the reagents may also be used to vary the thickness and permeability of the capsule shell.

A species, or other partition such as a droplet, may be encapsulated by, for example, including the species in the first fluid. Including the species in the second fluid may embed the species in the shell of the capsule. Of course, depending on the needs of the particular sample processing method, the phases may also be reversed—i.e., the first phase may be an oil phase and the second phase may be an aqueous phase.

2. Production of Capsules within Capsules by Flow Focusing

In some cases, capsules within capsules may be produced by flow focusing. With reference to FIG. **6**, a first (e.g., aqueous) fluid comprising a capsule, monomer, crosslinker, initiator, and aqueous surfactant **601** is flowed into a second (oil) fluid comprising a surfactant and an accelerator **602**. After entering the second fluid at a T-junction in a microfluidic device **603**, a droplet of first fluid breaks off from the first fluid stream and a second capsule shell begins to form around the capsule **604** due to the mixing of the monomer, crosslinker, and initiator in the first fluid and the accelerator in the second fluid. Thus, a capsule within a capsule is formed. As the capsule proceeds downstream, the shell becomes thicker due to increased exposure to the accelerator. Varying the concentrations of the reagents may also be used to vary the thickness and permeability of the second capsule shell.

A species may be encapsulated by, for example, including the species in the first fluid. Including the species in the second fluid may embed the species in the second shell of the capsule. Of course, depending on the needs of the particular sample processing method, the phases may also be reversed—i.e., the first phase may be an oil phase and the second phase may be an aqueous phase.

3. Production of Capsules in Batch

In some cases, capsules may be produced in batch, using capsule precursors, such as the droplets in an emulsion. With reference to FIG. **7**, capsule precursors **701** may be formed by any suitable method, for example by producing an emulsion with droplets comprising a monomer, a crosslinker, an initiator, and a surfactant. An accelerator may then be added to the medium, resulting in the formation of capsules **702**. As for the methods of flow focusing, the thickness of the shell can be varied by varying the concentrations of the reactants, and the time of exposure to the accelerator. The capsules may then be washed and recovered. As for any method described herein, a species, including other partitions, may be encapsulated within the capsule or, if suitable, within the shell.

In another example, the droplets of an emulsion may be exposed to an accelerator that is present in an outlet well during the emulsion generation process. For example, capsule precursors may be formed by any suitable method, such as the flow focusing method illustrated in FIG. **5**. Rather than including the accelerator in second fluid **502**, the accelerator may be included in a medium located at the exit of the T-junction (e.g., a medium located at the far-right of the horizontal channel of FIG. **5**. As the emulsion droplets (i.e., capsule precursors) exit the channel, they contact the medium comprising the accelerator (i.e., the outlet medium). If the capsule precursor has a density that is less than the density of outlet medium, the capsule precursors will rise through the medium, ensuring convectional and diffusional

exposure to the accelerator and reducing the likelihood of polymerization at the outlet of the channel.

III. SPECIES

The methods, compositions, systems, devices, and kits of this disclosure may be used with any suitable species. A species can be, for example, any substance used in sample processing, such as a reagent or an analyte. Exemplary species include whole cells, chromosomes, polynucleotides, organic molecules, proteins, polypeptides, carbohydrates, saccharides, sugars, lipids, enzymes, restriction enzymes, ligases, polymerases, barcodes, adapters, small molecules, antibodies, fluorophores, deoxynucleotide triphosphates (dNTPs), dideoxynucleotide triphosphates (ddNTPs), buffers, acidic solutions, basic solutions, temperature-sensitive enzymes, pH-sensitive enzymes, light-sensitive enzymes, metals, metal ions, magnesium chloride, sodium chloride, manganese, aqueous buffer, mild buffer, ionic buffer, inhibitors, saccharides, oils, salts, ions, detergents, ionic detergents, non-ionic detergents, oligonucleotides, nucleotides, DNA, RNA, peptide polynucleotides, complementary DNA (cDNA), double stranded DNA (dsDNA), single stranded DNA (ssDNA), plasmid DNA, cosmid DNA, chromosomal DNA, genomic DNA, viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), mRNA, rRNA, tRNA, nRNA, siRNA, snRNA, snoRNA, scaRNA, microRNA, dsRNA, ribozyme, riboswitch and viral RNA, a locked nucleic acid (LNA) in whole or part, locked nucleic acid nucleotides, any other type of nucleic acid analogue, proteases, nucleases, protease inhibitors, nuclease inhibitors, chelating agents, reducing agents, oxidizing agents, probes, chromophores, dyes, organics, emulsifiers, surfactants, stabilizers, polymers, water, small molecules, pharmaceuticals, radioactive molecules, preservatives, antibiotics, aptamers, and the like. In summary, the species that are used will vary depending on the particular sample processing needs.

In some cases, a partition comprises a set of species that have a similar attribute (e.g., a set of enzymes, a set of minerals, a set of oligonucleotides, a mixture of different barcodes, a mixture of identical barcodes). In other cases, a partition comprises a heterogeneous mixture of species. In some cases, the heterogeneous mixture of species comprises all components necessary to perform a particular reaction. In some cases, such mixture comprises all components necessary to perform a reaction, except for 1, 2, 3, 4, 5, or more components necessary to perform the reaction. In some cases, such additional components are contained within a different partition or within a solution within or surrounding a partition.

A species may be naturally-occurring or synthetic. A species may be present in a sample obtained using any methods known in the art. In some cases, a sample may be processed before analyzing it for an analyte.

A species may be obtained from any suitable location, including from organisms, whole cells, cell preparations and cell-free compositions from any organism, tissue, cell, or environment. A species may be obtained from environmental samples, biopsies, aspirates, formalin fixed embedded tissues, air, agricultural samples, soil samples, petroleum samples, water samples, or dust samples. In some instances, a species may be obtained from bodily fluids which may include blood, urine, feces, serum, lymph, saliva, mucosal secretions, perspiration, central nervous system fluid, vaginal fluid, or semen. Species may also be obtained from manufactured products, such as cosmetics, foods, personal care products, and the like. Species may be the products of experimental manipulation including, recombinant cloning, polynucleotide amplification, polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

In some cases, a species may quantified by mass. A species may be provided in a mass of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000 ng, 1 μg, 5 μg, 10 μg, 15 μg, or 20 μg. A species may be provided in a mass of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000 ng, 1 μg, 5 μg, 10 μg, 15 μg, or 20 μg. A species may be provided in a mass of less than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000 ng 1 μg, 5 μg, 10 μg, 15 μg, or 20 μg. A species may be provided in a mass ranging from about 1-10, 10-50, 50-100, 100-200, 200-1000, 1000-10000 ng, 1-5 or 1-20 μg. As described elsewhere in this disclosure, if a species is a polynucleotide, amplification may be used to increase the quantity of a polynucleotide.

Polynucleotides may also be quantified as "genome equivalents." A genome equivalent is an amount of polynucleotide equivalent to one haploid genome of an organism from which the target polynucleotide is derived. For example, a single diploid cell contains two genome equivalents of DNA. Polynucleotides may be provided in an amount ranging from about 1-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, or 100000-1000000 genome equivalents. Polynucleotides may be provided in an amount of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, 20000, 30000, 40000, 50000, 60000 70000, 80000, 90000, 100000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, or 1000000 genome equivalents. Polynucleotides may be provided in an amount less than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, 20000, 30000, 40000, 50000, 60000 70000, 80000, 90000, 100000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, or 1000000 genome equivalents.

Polynucleotides may also be quantified by the amount of sequence coverage provided. The amount of sequence coverage refers to the average number of reads representing a given nucleotide in a reconstructed sequence. Generally, the greater the number of times a region is sequenced, the more accurate the sequence information obtained. Polynucleotides may be provided in an amount that provides a range of sequence coverage from about 0.1X-10X, 10-X-50X, 50X-100X, 100X-200X, or 200X-500X. Polynucleotides may be provided in an amount that provides at least about 0.1X, 0.2X, 0.3X, 0.4X, 0.5X, 0.6X, 0.7X, 0.8X, 0.9X, 1.0X, 5X, 10X, 25X, 50X, 100X, 125X, 150X, 175X, or 200X sequence coverage. Polynucleotides may be provided in an amount that provides less than about 0.2X, 0.3X, 0.4X, 0.5X, 0.6X, 0.7X, 0.8X, 0.9X, 1.0X, 5X, 10X, 25X, 50X, 100X, 125X, 150X, 175X, or 200X sequence coverage.

In some cases, species are introduced into a partition either before or after a particular step. For example, a lysis buffer reagent may be introduced into a partition following

partitioning of a cellular sample into the partitions. In some cases, reagents and/or partitions comprising reagents are introduced sequentially such that different reactions or operations occur at different steps. The reagents (or partitions comprising reagents) may be also be loaded at steps interspersed with a reaction or operation step. For example, capsules comprising reagents for fragmenting molecules (e.g., nucleic acids) may be loaded into a well, followed by a fragmentation step, which may be followed by loading of capsules comprising reagents for ligating barcodes (or other unique identifiers, e.g., antibodies) and subsequent ligation of the barcodes to the fragmented molecules.

IV. PROCESSING OF ANALYTES AND OTHER SPECIES

In some cases, the methods, compositions, systems, devices, and kits of this disclosure may be used to process a sample containing a species, for example an analyte. Any suitable process can be performed.

a. Fragmentation of Target Polynucleotides

In some cases, the methods, compositions, systems, devices, and kits of this disclosure may be used for polynucleotide fragmentation. Fragmentation of polynucleotides is used as a step in a variety of methods, including polynucleotide sequencing. The size of the polynucleotide fragments, typically described in terms of length (quantified by the linear number of nucleotides per fragment), may vary depending on the source of the target polynucleotide, the method used for fragmentation, and the desired application. A single fragmentation step or a plurality of fragmentation steps may be used.

Fragments generated using the methods described herein may be about 1-10, 10-20, 20-50, 50-100, 50-200, 100-200, 200-300, 300-400, 400-500, 500-1000, 1000-5000, 5000-10000, 10000-100000, 100000-250000, or 250000-500000 nucleotides in length. Fragments generated using the methods described herein may be at least about 10, 20, 100, 200, 300, 400, 500, 1000, 5000, 10000, 100000, 250000, 500000, or more nucleotides in length. Fragments generated using the methods described herein may be less than about 10, 20, 100, 200, 300, 400, 500, 1000, 5000, 10000, 100000, 250000, 500000, nucleotides in length.

Fragments generated using the methods described herein may have a mean or median length of about 1-10, 10-20, 20-50, 50-100, 50-200, 100-200, 200-300, 300-400, 400-500, 500-1000, 1000-5000, 5000-10000, 10000-100000, 100000-250000, or 250000-500000 nucleotides. Fragments generated using the methods described herein may have a mean or median length of at least about 10, 20, 100, 200, 300, 400, 500, 1000, 5000, 10000, 100000, 250000, 500000, or more nucleotides. Fragments generated using the methods described herein may have a mean or median length of less than about 10, 20, 100, 200, 300, 400, 500, 1000, 5000, 10000, 100000, 250000, 500000, nucleotides.

Numerous fragmentation methods are known in the art. For example, fragmentation may be performed through physical, mechanical or enzymatic methods. Physical fragmentation may include exposing a target polynucleotide to heat or to UV light. Mechanical disruption may be used to mechanically shear a target polynucleotide into fragments of the desired range. Mechanical shearing may be accomplished through a number of methods known in the art, including repetitive pipetting of the target polynucleotide, sonication (e.g., using ultrasonic waves), cavitation and nebulization. Target polynucleotides may also be fragmented using enzymatic methods. In some cases, enzymatic digestion may be performed using enzymes such as using restriction enzymes.

While the methods of fragmentation described in the preceding paragraph, and in some paragraphs of the disclosure, are described with reference to "target" polynucleotides, this is not meant to be limiting, above or anywhere else in this disclosure. Any method of fragmentation described herein, or known in the art, can be applied to any polynucleotide used with the invention. In some cases, this polynucleotide may be a target polynucleotide, such as a genome. In other cases, this polynucleotide may be a fragment of a target polynucleotide which one wishes to further fragment. In still other cases, still further fragments may be still further fragmented. Any suitable polynucleotide may be fragmented according the methods described herein.

Restriction enzymes may be used to perform specific or non-specific fragmentation of target polynucleotides. The methods of the present disclosure may use one or more types of restriction enzymes, generally described as Type I enzymes, Type II enzymes, and/or Type III enzymes. Type II and Type III enzymes are generally commercially available and well known in the art. Type II and Type III enzymes recognize specific sequences of nucleotide base pairs within a double stranded polynucleotide sequence (a "recognition sequence" or "recognition site"). Upon binding and recognition of these sequences, Type II and Type III enzymes cleave the polynucleotide sequence. In some cases, cleavage will result in a polynucleotide fragment with a portion of overhanging single stranded DNA, called a "sticky end." In other cases, cleavage will not result in a fragment with an overhang, creating a "blunt end." The methods of the present disclosure may comprise use of restriction enzymes that generate either sticky ends or blunt ends.

Restriction enzymes may recognize a variety of recognition sites in the target polynucleotide. Some restriction enzymes ("exact cutters") recognize only a single recognition site (e.g., GAATTC). Other restriction enzymes are more promiscuous, and recognize more than one recognition site, or a variety of recognition sites. Some enzymes cut at a single position within the recognition site, while others may cut at multiple positions. Some enzymes cut at the same position within the recognition site, while others cut at variable positions.

The present disclosure provides method of selecting one or more restriction enzymes to produce fragments of a desired length. Polynucleotide fragmentation may be simulated in silico, and the fragmentation may be optimized to obtain the greatest number or fraction of polynucleotide fragments within a particular size range, while minimizing the number or fraction of fragments within undesirable size ranges. Optimization algorithms may be applied to select a combination of two or more enzymes to produce the desired fragment sizes with the desired distribution of fragments quantities.

A polynucleotide may be exposed to two or more restriction enzymes simultaneously or sequentially. This may be accomplished by, for example, adding more than one restriction enzyme to a partition, or by adding one restriction enzyme to a partition, performing the digestion, deactivating the restriction enzyme (e.g., by heat treatment) and then adding a second restriction enzyme. Any suitable restriction enzyme may be used alone, or in combination, in the methods presented herein.

In some cases, a species is a restriction enzyme that is a "rare-cutter." The term "rare-cutter enzyme," as used herein, generally refers to an enzyme with a recognition site that

occurs only rarely in a genome. The size of restriction fragments generated by cutting a hypothetical random genome with a restriction enzyme may be approximated by 4^N, where N is the number of nucleotides in the recognition site of the enzyme. For example, an enzyme with a recognition site consisting of 7 nucleotides would cut a genome once every 4^7 bp, producing fragments of about 16,384 bp. Generally rare-cutter enzymes have recognition sites comprising 6 or more nucleotides. For example, a rare cutter enzyme may have a recognition site comprising or consisting of 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 nucleotides. Examples of rare-cutter enzymes include NotI (GCGGCCGC), XmaIII (CGGCCG), SstII (CCGCGG), SalI (GTCGAC), NruI (TCGCGA), NheI (GCTAGC), Nb.BbvCI (CCTCAGC), BbvCI (CCTCAGC), AscI (GGCGCGCC), AsiSI (GCGATCGC), FseI (GGCCGGCC), PacI (TTAATTAA), PmeI (GTTTAAAC), SbfI (CCTGCAGG), SgrAI (CRCCGGYG), SwaI (ATTTAAAT), BspQI (GCTCTTC), SapI (GCTCTTC), SfiI (GGCCNNNNNGGCC), CspCI (CAANNNNNGTGG), AbsI (CCTCGAGG), CciNI (GCGGCCGC), FspAI (RTGCGCAY), MauBI (CGCGCGCG), MreI (CGCCGGCG), MssI (GTTTAAAC), PalAI (GGCGCGCC), RgaI (GCGATCGC), RigI (GGCCGGCC), SdaI (CCTGCAGG), SfaAI (GCGATCGC), SgfI (GCGATCGC), SgrDI (CGTCGACG), SgsI (GGCGCGCC), SmiI (ATTTAAAT), SrfI (GCCCGGGC), Sse2321 (CGCCGGCG), Sse83871 (CCTGCAGG), LguI (GCTCTTC), PciSI (GCTCTTC), AarI (CACCTGC), AjuI (GAANNNNNNNTTGG), AloI (GAACNNNNNNTCC), BarI (GAAGNNNNNNTAC), PpiI (GAACNNNNNCTC), PsrI (GAACNNNNNNTAC), and others.

In some cases, polynucleotides may be fragmented and barcoded at the same time. For example, a transposase (e.g., NEXTERA) may be used to fragment a polynucleotide and add a barcode to the polynucleotide.

b. Barcoding

Certain downstream applications, for example polynucleotide sequencing, may rely on the barcodes to identify the origin of a sequence and, for example, to assemble a larger sequence from sequenced fragments. Therefore, it may be desirable to add barcodes to polynucleotide fragments before sequencing. Barcodes may be of a variety of different formats, including polynucleotide barcodes. Depending upon the specific application, barcodes may be attached to polynucleotide fragments in a reversible or irreversible manner. Barcodes may also allow for identification and/or quantification of individual polynucleotide fragments during sequencing.

Barcodes may be loaded into partitions so that one or more barcodes are introduced into a particular partition. Each partition may contain a different set of barcodes. In some cases, each different set of barcodes may comprise a set of identical barcodes. This may be accomplished by directly dispensing the barcodes into the partitions, or by placing the barcodes within a partition within a partition.

The number of different barcodes or different sets of barcodes that are partitioned may vary depending upon, for example, the particular barcodes to be partitioned and/or the application. Different sets of barcodes may be, for example, sets of identical barcodes where the identical barcodes differ between each set. Or different sets of barcodes may be, for example, sets of different barcodes, where each set differs in its included barcodes. For example, about 1, 5, 10, 50, 100, 1000, 10000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, 100000000, or more different barcodes or different sets of barcodes may be partitioned. In some examples, at least about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, 100000000, or more different barcodes or different sets of barcodes may be partitioned. In some examples, less than about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 different barcodes or different sets of barcodes may be partitioned. In some examples, about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, 100000-1000000, 10000-1000000, 10000-10000000, or 10000-100000000 barcodes may be partitioned.

Barcodes may be partitioned at a particular density. For example, barcodes may be partitioned so that each partition contains about 1, 5, 10, 50, 100, 1000, 10000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 barcodes per partition. Barcodes may be partitioned so that each partition contains at least about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, 100000000, or more barcodes per partition. Barcodes may be partitioned so that each partition contains less than about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 barcodes per partition. Barcodes may be partitioned such that each partition contains about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, 100000-1000000, 10000-1000000, 10000-10000000, or 10000-100000000 barcodes per partition.

Barcodes may be partitioned such that identical barcodes are partitioned at a particular density. For example, identical barcodes may be partitioned so that each partition contains about 1, 5, 10, 50, 100, 1000, 10000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 identical barcodes per partition. Barcodes may be partitioned so that each partition contains at least about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, 100000000, or more identical barcodes per partition. Barcodes may be partitioned so that each partition contains less than about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000,

90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 identical barcodes per partition. Barcodes may be partitioned such that each partition contains about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, 100000-1000000, 10000-1000000, 10000-10000000, or 10000-100000000 identical barcodes per partition.

Barcodes may be partitioned such that different barcodes are partitioned at a particular density. For example, different barcodes may be partitioned so that each partition contains about 1, 5, 10, 50, 100, 1000, 10000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 different barcodes per partition. Barcodes may be partitioned so that each partition contains at least about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, 100000000, or more different barcodes per partition. Barcodes may be partitioned so that each partition contains less than about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 different barcodes per partition. Barcodes may be partitioned such that each partition contains about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, 100000-1000000, 10000-1000000, 10000-10000000, or 10000-100000000 different barcodes per partition.

The number of partitions employed to partition barcodes may vary, for example, depending on the application and/or the number of different barcodes to be partitioned. For example, the number of partitions employed to partition barcodes may be about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, or 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100,000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1,000,000, 2000000, 3000000, 4000000, 5000000, 10000000, 20000000 or more. The number of partitions employed to partition barcodes may be at least about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1000000, 2000000, 3000000, 4000000, 5000000, 10000000, 20000000 or more. The number of partitions employed to partition barcodes may be less than about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1000000, 2000000, 3000000, 4000000, 5000000, 10000000, or 20000000. The number of partitions employed to partition barcodes may be about 5-10000000, 5-5000000, 5-1,000,000, 10-10,000, 10-5,000, 10-1,000, 1,000-6,000, 1,000-5,000, 1,000-4,000, 1,000-3,000, or 1,000-2,000.

As described above, different barcodes or different sets of barcodes (e.g., each set comprising a plurality of identical barcodes or different barcodes) may be partitioned such that each partition comprises a different barcode or different barcode set. In some cases, each partition may comprise a different set of identical barcodes. Where different sets of identical barcodes are partitioned, the number of identical barcodes per partition may vary. For example, about 100,000 or more different sets of identical barcodes may be partitioned across about 100,000 or more different partitions, such that each partition comprises a different set of identical barcodes. In each partition, the number of identical barcodes per set of barcodes may be about 1,000,000 identical barcodes. In some cases, the number of different sets of barcodes may be equal to or substantially equal to the number of partitions. Any suitable number of different barcodes or different barcode sets (including numbers of different barcodes or different barcode sets to be partitioned described elsewhere herein), number of barcodes per partition (including numbers of barcodes per partition described elsewhere herein), and number of partitions (including numbers of partitions described elsewhere herein) may be combined to generate a diverse library of partitioned barcodes with high numbers of barcodes per partition. Thus, as will be appreciated, any of the above-described different numbers of barcodes may be provided with any of the above-described barcode densities per partition, and in any of the above-described numbers of partitions.

The barcodes may be loaded into the partitions at an expected or predicted ratio of barcodes per species to be barcoded (e.g., polynucleotide fragment, strand of polynucleotide, cell, etc.). In some cases, the barcodes are loaded into partitions such that more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 barcodes are loaded per species. In some cases, the barcodes are loaded in the partitions so that less than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 barcodes are loaded per species. In some cases, the average number of barcodes loaded per species is less than, or greater than, about 0.0001, 0.001, 0.01, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 barcodes per species.

When more than one barcode is present per polynucleotide fragment, such barcodes may be copies of the same barcode, or multiple different barcodes. For example, the attachment process may be designed to attach multiple identical barcodes to a single polynucleotide fragment, or multiple different barcodes to the polynucleotide fragment.

The methods provided herein may comprise loading a partition with the reagents necessary for the attachment of barcodes to polynucleotide fragments. In the case of ligation reactions, reagents including restriction enzymes, ligase enzymes, buffers, adapters, barcodes and the like may be loaded into a partition. In the case barcoding by amplification, reagents including primers, DNA polymerases, DNTPs, buffers, barcodes and the like may be loaded into a partition. As described throughout this disclosure, these reagents may be loaded directly into the partition, or via another partition.

Barcodes may be ligated to a polynucleotide fragment using sticky or blunt ends. Barcoded polynucleotide fragments may also be generated by amplifying a polynucleotide fragment with primers comprising barcodes. As with any other species discussed in this disclosure, these modules may be contained within the same or different partitions, depending on the needs of assay or process.

Barcodes may be assembled combinatorially, from smaller components designed to assemble in a modular format. For example, three modules, 1A, 1B, and 1C may be combinatorially assembled to produce barcode 1ABC. Such combinatorial assembly may significantly reduce the cost of

synthesizing a plurality of barcodes. For example, a combinatorial system consisting of 3 A modules, 3 B modules, and 3 C modules may generate 3*3*3=27 possible barcode sequences from only 9 modules.

In some cases, barcodes may be combinatorially assembled by mixing two oligonucleotides and hybridizing them to produce annealed or partially annealed oligonucleotides (e.g., forked adapters). These barcodes may comprise an overhang of one or more nucleotides, in order to facilitate ligation with polynucleotide fragments that are to be barcoded. In some cases, the 5' end of the antisense strand may be phosphorylated in order to ensure double-stranded ligation. Using this approach, different modules may be assembled by, for example, mixing oligonucleotides A and B, A and C, A and D, B and C, B, and D, and so on. The annealed oligonucleotides may also be synthesized as a single molecule with a hairpin loop that may be cut after ligation to the polynucleotide to be barcoded.

c. Amplification

In some cases, a polynucleotide is amplified during sample processing. This amplification may be performed in one or more of the partitions described in this disclosure. Amplification may be useful for a variety of purposes, including but not limited to generating multiple copies of polynucleotide sequences, addition of adapter sequences or barcodes to polynucleotides, mutation or error detection, producing higher quality samples for further downstream processing and the like.

An suitable amplification method may be utilized, including polymerase chain reaction (PCR), ligase chain reaction (LCR), helicase-dependent amplification, linear after the exponential PCR (LATE-PCR) asymmetric amplification, digital PCR, degenerate oligonucleotide primer PCR (DOP-PCR), primer extension pre-amplification PCR (PEP-PCR) and ligation mediated PCR, rolling circle amplification, multiple displacement amplification (MDA), and single primer isothermal linear amplification. In one example, MDA may be performed on a species contained within a partition. In some cases, this species is a whole chromosome from a cell.

V. STIMULI-RESPONSIVENESS

In some cases, stimuli may be used to trigger the release of a species from a partition. Generally, a stimulus may cause disruption of the structure of a partition, such as the wall of a well, a component of a spot, the stability of a droplet (e.g., a droplet in an emulsion), or the shell of a capsule. These stimuli are particularly useful in inducing a partition to release its contents. Because a partition may be contained within another partition, and each partition may be responsive (or not responsive) to different stimuli, stimuli-responsiveness may be employed to release the contents of one partition (e.g., a partition responsive to the stimulus) into another partition (e.g., a partition not responsive to that stimulus, or less responsive to that stimulus).

FIG. **8**A shows one non-limiting example using a capsule as an exemplary partition. More specifically, FIG. **8**A shows selective release of the contents of an inner capsule **801** into the contents of an outer capsule **802** by applying a stimulus that dissolves the inner capsule **803**, resulting in a capsule containing a mixed sample **804**.

FIG. **8**B shows another non-limiting example using a multiple partitions in a partition scheme. An outer partition **805** comprises inner partitions **806** and **807**. Inner partition **806** comprises species A and inner partition **807** comprises species B along with its own inner partition **809** comprising species C. For example, the two inner partitions **806** and **807** may be droplets of an emulsion and outer partition **805** comprising the two droplets may be a well. In some cases, one or both of the two droplets may be a micelle Inner partition **809** may also be a droplet of an emulsion and may be capable of being dissolved or degraded by species A. In some cases species A may be chemical stimulus, such as, for example, a reducing agent (e.g., DTT, TCEP, etc.) capable of breaking disulfide bonds and inner partition **809** may comprise disulfide bonds (e.g., a gel bead comprising disulfide bonds) capable of being broken by species A.

Via diffusion or other means, inner partitions **806** and **807** may come into contact and fuse **812** together such that a new partition **808** can be generated comprising the combined contents of inner partitions **806** and **807** (e.g., species A+B+inner partition **809**). The action **810** of species A on inner partition **809** can degrade or dissolve inner partition **809** such that the contents (e.g., species C) of inner partition **809** can be released into partition **808** to generate a new partition **811**. Partition **811** comprises the combined contents of inner partitions **806**, **807**, and **809** (e.g., species A+B+C).

FIG. **8**C shows another non-limiting example using a multiple partitions in a partition scheme. Outer partition **814** may comprise inner partition **815** and one or more inner partitions **816** (multiple inner partitions **816** are shown) and may be, for example, a droplet of an emulsion. Inner partition **815** comprises species A and inner partition **816** comprises species B. In some cases, species B may be a chemical stimulus capable of degrading or dissolving inner partition **815**. Species B may be, for example, a reducing agent (e.g., DTT, TCEP, etc.) capable of breaking disulfide bonds and inner partition **815** may comprise disulfide bonds (e.g., a gel bead comprising disulfide bonds) capable of being dissolved or degraded by species B. Moreover, inner partition **816** may be heat sensitive (e.g., a paraffin or other wax bead) such that upon application of heat, species B can be released into the interior of outer partition **814**.

Upon application of an appropriate stimulus **817** (e.g., heat), inner partitions **816** may be disrupted or degraded such that species B is released to the interior of outer partition **814**. The action **818** of species B on inner partition **815** can degrade or dissolve inner partition **815** such that the contents of inner partition **815** can be released to the interior of outer partition **814**. Outer partition **814** can then comprise the combined contents (e.g., species A+B) of inner partitions **815** and **816**.

In another example, species B as described above with respect to FIG. **8**C, may be a reagent necessary to start a reaction in the interior of outer partition **814**, such as, for example, an amplification reaction. Upon degradation or disruption of inner partition **816** with the appropriate stimulus (e.g., heat), species B may be released to the interior of outer partition **814**, and the desired reaction allowed to commence with or without the application of an additional stimulus.

FIG. **8**D shows another non-limiting example using a multiple partitions in a partition scheme. Outer partition **819** comprises species A and inner partition **820** and may be, for example, a droplet of an emulsion. Inner partition **820** can be impermeable to species A Inner partition **820** can comprise its own inner partition **821** which comprises species B Inner partition **820** may be sensitive to a stimulus such that when the stimulus is applied to inner partition **820**, inner partition **820** is dissolved or degraded and inner partition **821** is released to the interior of outer partition **819**. Inner partition **820** may be, for example, a microcapsule with a hardened shell that comprises, for example, a heat-sensitive shell that

degrades or melts when heat is applied to inner partition **820**. Inner partition **821** may be sensitive to species A, such that species A is capable of degrading or dissolving inner partition **821**. For example, species A may be a reducing agent capable of breaking disulfide bonds and inner partition **821** may comprise a species comprising disulfide bonds (e.g., a gel bead comprising disulfide bonds) capable of being broken with species A.

Upon application of an appropriate stimulus **822** (e.g., heat), inner partition **820** may be disrupted or degraded such that inner partition **821** is released to the interior of outer partition **819**. The action **823** of species A on inner partition **821** can degrade or dissolve inner partition **821** such that the contents of inner partition **821** (e.g., species B) can be released to the interior of outer partition **819**. Outer partition **819** can then comprise the combined contents (e.g., species A+B) of inner partition **821** and outer partition **819**.

Of course, the configuration shown in FIGS. **8**A-**8**D are merely illustrative, and stimuli-responsiveness may be used to release the contents of any suitable partition into any other suitable partition, medium, or container (see, e.g., Table 1 for more specific examples of partitions within partitions).

Examples of stimuli that may be used include chemical stimuli, bulk changes, biological stimuli, light, thermal stimuli, magnetic stimuli, addition of a medium to a well, and any combination thereof, as described more fully below. (See, e.g., Esser-Kahn et al., (2011) *Macromolecules* 44: 5539-5553; Wang et al., (2009) *Chem Phys Chem* 10:2405-2409.)

a. Chemical Stimuli and Bulk Changes

Numerous chemical triggers may be used to trigger the disruption of partitions (e.g., Plunkett et al., Biomacromolecules, 2005, 6:632-637). Examples of these chemical changes may include, but are not limited to pH-mediated changes to the integrity of a component of a partition, disintegration of a component of a partition via chemical cleavage of crosslink bonds, and triggered depolymerization of a component of a partition. Bulk changes may also be used to trigger disruption of partitions.

A change in pH of a solution, such as a decrease in pH, may trigger disruption of a partition via a number of different mechanisms. The addition of acid may cause degradation or disassembly a portion of a partition through a variety of mechanisms. Addition of protons may disassemble cross-linking of polymers in a component of a partition, disrupt ionic or hydrogen bonds in a component of a partition, or create nanopores in a component of a partition to allow the inner contents to leak through to the exterior. A change in pH may also destabilize an emulsion, leading to release of the contents of the droplets.

In some examples, a partition is produced from materials that comprise acid-degradable chemical cross-linkers, such a ketals. A decrease in pH, particular to a pH lower than 5, may induce the ketal to convert to a ketone and two alcohols and facilitate disruption of the partition. In other examples, the partitions may be produced from materials comprising one or more polyelectrolytes that are pH sensitive. A decrease in pH may disrupt the ionic- or hydrogen-bonding interactions of such partitions, or create nanopores therein. In some cases, partitions made from materials comprising polyelectrolytes comprise a charged, gel-based core that expands and contracts upon a change of pH.

Disruption of cross-linked materials comprising a partition can be accomplished through a number of mechanisms. In some examples, a partition can be contacted with various chemicals that induce oxidation, reduction or other chemical changes. In some cases, a reducing agent, such as beta-mercaptoethanol, can be used, such that disulfide bonds of a partition are disrupted. In addition, enzymes may be added to cleave peptide bonds in materials forming a partition, thereby resulting in a loss of integrity of the partition.

Depolymerization can also be used to disrupt partitions. A chemical trigger may be added to facilitate the removal of a protecting head group. For example, the trigger may cause removal of a head group of a carbonate ester or carbamate within a polymer, which in turn causes depolymerization and release of species from the inside of a partition.

In yet another example, a chemical trigger may comprise an osmotic trigger, whereby a change in ion or solute concentration in a solution induces swelling of a material used to make a partition. Swelling may cause a buildup of internal pressure such that a partition ruptures to release its contents. Swelling may also cause an increase in the pore size of the material, allowing species contained within the partition to diffuse out, and vice versa.

A partition may also be made to release its contents via bulk or physical changes, such as pressure induced rupture, melting, or changes in porosity.

b. Biological Stimuli

Biological stimuli may also be used to trigger disruption of partitions. Generally, biological triggers resemble chemical triggers, but many examples use biomolecules, or molecules commonly found in living systems such as enzymes, peptides, saccharides, fatty acids, nucleic acids and the like. For example, partitions may be made from materials comprising polymers with peptide cross-links that are sensitive to cleavage by specific proteases. More specifically, one example may comprise a partition made from materials comprising GFLGK peptide cross links. Upon addition of a biological trigger such as the protease Cathepsin B, the peptide cross links of the shell well are cleaved and the contents of the capsule are released. In other cases, the proteases may be heat-activated. In another example, partitions comprise a component comprising cellulose. Addition of the hydrolytic enzyme chitosan serves as biologic trigger for cleavage of cellulosic bonds, depolymerization of component of the partition comprising chitosan, and release of its inner contents.

c. Thermal Stimuli

Partitions may also be induced to release their contents upon the application of a thermal stimulus. A change in temperature can cause a variety changes to a partition. A change in heat may cause melting of a partition such that a portion of the partition disintegrates, or disruption of an emulsion. In other cases, heat may increase the internal pressure of the inner components of a partition such that the partition ruptures or explodes. In still other cases, heat may transform a partition into a shrunken dehydrated state. Heat may also act upon heat-sensitive polymers used as materials to construct partitions.

In one example, a partition is made from materials comprising a thermo-sensitive hydrogel. Upon the application of heat, such as a temperature above 35 C, the hydrogel material shrinks. The sudden shrinkage of the material increases the pressure and ruptures the partition.

In some cases, a material used to produce a partition may comprise a diblock polymer, or a mixture of two polymers, with different heat sensitivities. One polymer may be particularly likely to shrink after the application of heat, while the other is more heat-stable. When heat is applied to such shell wall, the heat-sensitive polymer may shrink, while the other remains intact, causing a pore to form. In still other cases, a material used to produce a partition may comprise

magnetic nanoparticles. Exposure to a magnetic field may cause the generation of heat, leading to rupture of the partition.

d. Magnetic Stimuli

Inclusion of magnetic nanoparticles in a material used to produce a partition may allow triggered rupture of the partition, as described above, as well as enable guidance of these partitions to other partitions (e.g., guidance of capsules to wells in an array). In one example, incorporation of Fe_3O_4 nanoparticles into materials used to produce partitions triggers rupture in the presence of an oscillating magnetic field stimulus.

e. Electrical and Light Stimuli

A partition may also be disrupted as the result of electrical stimulation. Similar to the magnetic particles described in the previous section, electrically sensitive particles can allow for both triggered rupture of partitions, as well as other functions such as alignment in an electric field or redox reactions. In one example, partitions made from materials comprising electrically sensitive material are aligned in an electric field such that release of inner reagents can be controlled. In other examples, electric fields may induce redox reactions within a partition that may increase porosity.

A light stimulus may also be used to disrupt the partitions. Numerous light triggers are possible and may include systems that use various molecules such as nanoparticles and chromophores capable of absorbing photons of specific ranges of wavelengths. For example, metal oxide coatings can be used to produce certain partitions. UV irradiation of partitions coated with SiO2/TiO2 may result in disintegration of the partition wall. In yet another example, photo switchable materials such as azobenzene groups may be incorporated in the materials used to produce the partitions. Upon the application of UV or visible light, chemicals such as these undergo a reversible cis-to-trans isomerization upon absorption of photons. In this aspect, incorporation of photo switches results in disintegration of a portion of a partition, or an increase in porosity of a portion of a partition.

f. Application of Stimuli

The devices, methods, compositions, systems, and kits of this disclosure may be used in combination with any apparatus or device that provides such trigger or stimulus. For example, if the stimulus is thermal, a device may be used in combination with a heated or thermally controlled plate, which allows heating of the wells and may induce the rupture of capsules. Any of a number of methods of heat transfer may be used for thermal stimuli, including but not limited to applying heat by radiative heat transfer, convective heat transfer, or conductive heat transfer. In other cases, if the stimulus is a biological enzyme, the enzyme may be injected into a device such that it is deposited into each well. In another aspect, if the stimulus is a magnetic or electric field, a device may be used in combination with a magnetic or electric plate.

VI. APPLICATIONS

a. Polynucleotide Sequencing

Generally, the methods and compositions provided herein are useful for preparation of polynucleotide fragments for downstream applications such as sequencing. Sequencing may be performed by any available technique. For example, sequencing may be performed by the classic Sanger sequencing method. Sequencing methods may also include: high-throughput sequencing, pyrosequencing, sequencing-by-synthesis, single-molecule sequencing, nanopore sequencing, sequencing-by-ligation, sequencing-by-hybridization, RNA-Seq (Illumina), Digital Gene Expression (Helicos), next generation sequencing, single molecule sequencing by synthesis (SMSS) (Helicos), massively-parallel sequencing, clonal single molecule Array (Solexa), shotgun sequencing, SMRT sequencing (Pacific Biosciences) Maxim-Gilbert sequencing, primer walking, and any other sequencing methods known in the art.

In some cases varying numbers of fragments are sequenced. For example, in some cases about 30%-90% of the fragments are sequenced. In some cases, about 35%-85%, 40%-80%, 45%-75%, 50%-70%, 55%-65%, or 50%-60% of the fragments are sequenced. In some cases, at least about 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the fragments are sequenced. In some cases less than about 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the fragments are sequenced.

In some cases sequences from fragments are assembled to provide sequence information for a contiguous region of the original target polynucleotide that is longer than the individual sequence reads. Individual sequence reads may be about 10-50, 50-100, 100-200, 200-300, 300-400, or more nucleotides in length.

The identities of the barcode tags may serve to order the sequence reads from individual fragments as well as to differentiate between haplotypes. For example, during the partitioning of individual fragments, parental polynucleotide fragments may separated into different partitions. With an increase in the number of partitions, the likelihood of a fragment from both a maternal and paternal haplotype contained in the same partition becomes negligibly small. Thus, sequence reads from fragments in the same partition may be assembled and ordered.

b. Polynucleotide Phasing

This disclosure also provides methods and compositions to prepare polynucleotide fragments in such a manner that may enable phasing or linkage information to be generated. Such information may allow for the detection of linked genetic variations in sequences, including genetic variations (e.g., SNPs, mutations, indels, copy number variations, transversions, translocations, inversions, etc.) that are separated by long stretches of polynucleotides. The term "indel" refers to a mutation resulting in a colocalized insertion and deletion and a net gain or loss in nucleotides. A "microindel" is an indel that results in a net gain or loss of 1 to 50 nucleotides. These variations may exist in either a cis or trans relationship. In a cis relationship, two or more genetic variations exist in the same polynucleotide or strand. In a trans relationship, two or more genetic variations exist on multiple polynucleotide molecules or strands.

Methods provided herein may be used to determine polynucleotide phasing. For example, a polynucleotide sample (e.g., a polynucleotide that spans a given locus or loci) may be partitioned such that at most one molecule of polynucleotide is present per partition. The polynucleotide may then be fragmented, barcoded, and sequenced. The sequences may be examined for genetic variation. The detection of genetic variations in the same sequence tagged with two different bar codes may indicate that the two genetic variations are derived from two separate strands of DNA, reflecting a trans relationship. Conversely, the detection of two different genetic variations tagged with the same bar codes may indicate that the two genetic variations are from the same strand of DNA, reflecting a cis relationship.

Phase information may be important for the characterization of a polynucleotide fragment, particularly if the polynucleotide fragment is derived from a subject at risk of, having, or suspected of a having a particular disease or

disorder (e.g., hereditary recessive disease such as cystic fibrosis, cancer, etc.). The information may be able to distinguish between the following possibilities: (1) two genetic variations within the same gene on the same strand of DNA and (2) two genetic variations within the same gene but located on separate strands of DNA. Possibility (1) may indicate that one copy of the gene is normal and the individual is free of the disease, while possibility (2) may indicate that the individual has or will develop the disease, particularly if the two genetic variations are damaging to the function of the gene when present within the same gene copy. Similarly, the phasing information may also be able to distinguish between the following possibilities: (1) two genetic variations, each within a different gene on the same strand of DNA and (2) two genetic variations, each within a different gene but located on separate strands of DNA.

c. Sequencing Polynucleotides from Small Numbers of Cells

Methods provided herein may also be used to prepare polynucleotide contained within cells in a manner that enables cell-specific information to be obtained. The methods enable detection of genetic variations (e.g., SNPs, mutations, indels, copy number variations, transversions, translocations, inversions, etc.) from very small samples, such as from samples comprising about 10-100 cells. In some cases, about 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 cells may be used in the methods described herein. In some cases, at least about 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 cells may be used in the methods described herein. In other cases, at most about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 cells may be used in the methods described herein.

In an example, a method comprises partitioning a cellular sample (or crude cell extract) such that at most one cell (or extract of one cell) is present per partition, lysing the cells, fragmenting the polynucleotides contained within the cells by any of the methods described herein, attaching the fragmented polynucleotides to barcodes, pooling, and sequencing.

As described elsewhere herein, the barcodes and other reagents may be contained within a partition (e.g., a capsule). These capsules may be loaded into another partition (e.g., a well) before, after, or concurrently with the loading of the cell, such that each cell is contacted with a different capsule. This technique may be used to attach a unique barcode to polynucleotides obtained from each cell. The resulting tagged polynucleotides may then be pooled and sequenced, and the barcodes may be used to trace the origin of the polynucleotides. For example, polynucleotides with identical barcodes may be determined to originate from the same cell, while polynucleotides with different barcodes may be determined to originate from different cells.

The methods described herein may be used to detect the distribution of oncogenic mutations across a population of cancerous tumor cells. For example, some tumor cells may have a mutation, or amplification, of an oncogene (e.g., HER2, BRAF, EGFR, KRAS) in both alleles (homozygous), others may have a mutation in one allele (heterozygous), and still others may have no mutation (wild-type). The methods described herein may be used to detect these differences, and also to quantify the relative numbers of homozygous, heterozygous, and wild-type cells. Such information may be used, for example, to stage a particular cancer and/or to monitor the progression of the cancer and its treatment over time.

In some examples, this disclosure provides methods of identifying mutations in two different oncogenes (e.g., KRAS and EGFR). If the same cell comprises genes with both mutations, this may indicate a more aggressive form of cancer. In contrast, if the mutations are located in two different cells, this may indicate that the cancer is more benign, or less advanced.

d. Analysis of Gene Expression

Methods of the disclosure may be applicable to processing samples for the detection of changes in gene expression. A sample may comprise a cell, mRNA, or cDNA reverse transcribed from mRNA. The sample may be a pooled sample, comprising extracts from several different cells or tissues, or a sample comprising extracts from a single cell or tissue.

Cells may be placed directly into a partition (e.g., a microwell) and lysed. After lysis, the methods of the invention may be used to fragment and barcode the polynucleotides of the cell for sequencing. Polynucleotides may also be extracted from cells prior to introducing them into a partition used in a method of the invention. Reverse transcription of mRNA may be performed in a partition described herein, or outside of such a partition. Sequencing cDNA may provide an indication of the abundance of a particular transcript in a particular cell over time, or after exposure to a particular condition.

The methods presented above provide several advantages over current polynucleotide processing methods. First, inter-operator variability is greatly reduced. Second, the methods may be carried out in microfluidic devices, which have a low cost and can be easily fabricated. Third, the controlled fragmentation of the target polynucleotides allows the user to produce polynucleotide fragments with a defined and appropriate length. This aids in partitioning the polynucleotides and also reduces the amount of sequence information loss due to the present of overly-large fragments. The methods and systems also provide a facile workflow that maintains the integrity of the processed polynucleotide. Additionally, the use of restriction enzymes enables the user to create DNA overhangs ("sticky ends") that may be designed for compatibility with adapters and/or barcodes.

e. Partitioning of Polynucleotides, Such as Chromosomes, from Cells

In one example the methods, compositions, systems, devices, and kits provided in this disclosure may be used to partition polynucleotides, including whole chromosomes, from cells. In one example, a single cell or a plurality of cells (e.g., 2, 10, 50, 100, 1000, 10000, 25000, 50000, 100000, 500000, 1000000, or more cells) is loaded into a vessel with lysis buffer and proteinase K, and incubated for a specified period of time. Utilization of a plurality of cells will enable polynucleotide phasing, for example, by partitioning each polynucleotide to be analyzed in its own partition.

After incubation, the cell lysate is partitioned, for example by flow focusing the cell lysate into a capsule. If phasing is to be performed, flow focusing is performed such that each capsule comprises only a single analyte (e.g., a single chromosome), or only a single copy of any particular chromosome (e.g., one copy of a first chromosome and one copy of a second chromosome). In some cases, a plurality of chromosomes may be encapsulated within the same capsule, so long as the chromosomes are not the same chromosome. The encapsulation is performed under gentle flow, to minimize shearing of the polynucleotides. The capsule may be porous, to allow washing of the contents of the capsule, and introduction of reagents into the capsule, while maintaining the polynucleotides (e.g., chromosomes) within the capsules. The encapsulated polynucleotides (e.g., chromosomes) may then be processed according to any of the methods provided in this disclosure, or known in the art. The capsule shells protect the encapsulated polynucleotides (e.g.,

chromosomes) from shearing and further degradation. Of course, this method can also be applied to any other cellular component.

As described above, the capsule shell may be used to protect a polynucleotide from shearing. However, a capsule may also be used as a partition to enable compartmentalized shearing of a polynucleotide or other analyte. For example, in some cases a polynucleotide may be encapsulated within a capsule and then subject to ultrasonic shear, or any other suitable shearing. The capsule shell may be configured to remain intact under the shear, while the encapsulated polynucleotide may be sheared, but will remain within the capsule. In some cases, a hydrogel droplet may be used to accomplish the same end.

VIII. KITS

In some cases, this disclosure provides kits comprising reagents for the generation of partitions. The kit may comprise any suitable reagents and instructions for the generation of partitions and partitions within partitions.

In one example, a kit comprises reagents for generating capsules within droplets in an emulsion. For example, a kit may comprise reagents for generating capsules, reagents for generating an emulsion, and instructions for introducing the capsules into the droplets of the emulsion. As specified throughout this disclosure, any suitable species may be incorporated into the droplets and/or into the capsule. A kit of this disclosure may also provide any of these species. Similarly, as described throughout the disclosure, the capsule may be designed to release its contents into the droplets of the emulsion upon the application of a stimulus.

In another example, a kit comprises reagents for generating capsules within capsules. For example, a kit may comprise reagents for generating inner capsules, reagents for generating outer capsules, and instructions for generating capsules within capsules. As specified throughout this disclosure, any suitable species may be incorporated into the inner and/or outer capsules. A kit of this disclosure may also provide any of these species. Similarly, as described throughout the disclosure, the inner capsule may be designed to release its contents into the outer capsule upon the application of a stimulus.

IX. DEVICES

In some cases, this disclosure provides devices comprising partitions for the processing of analytes. A device may be a microwell array, or a microspot array, as described elsewhere in this disclosure. A device may formed in a manner that it comprises any suitable partition. In some cases, a device comprises a plurality of wells, or a plurality of spots. Of course, any partition in a device may also hold other partitions, such as a capsule, a droplet in an emulsion, and the like.

A device may be formed from any suitable material. In some examples, a device is formed from a material selected from the group consisting of fused silica, soda lime glass, borosilicate glass, poly(methyl methacrylate), sapphire, silicon, germanium, cyclic olefin copolymer, polyethylene, polypropylene, polyacrylate, polycarbonate, plastic, and combinations thereof.

In some cases, a device comprises channels for the flow of fluids into and between partitions. Any suitable channels may be used. A device may comprise a fluid inlet and a fluid outlet. The inlet and outlet may be attached to liquid handling devices to introduce species into the device. The device may be sealed, before or after introduction of any species.

Materials that are hydrophilic and/or hydrophobic may be used in different parts of the device. For example, in some cases a device of this disclosure comprises a partition with an interior surface comprising a hydrophilic material. In some cases a surface exterior to the partitions comprises a hydrophobic material. In some cases, a fluid flow path is coated with a hydrophobic or hydrophilic material.

As will be appreciated, the instant disclosure provides for the use of any of the compositions, methods, devices, and kits described herein for a particular use or purpose, including the various applications, uses, and purposes described herein. For example, the disclosure provides for the use of the compositions, methods, devices, and kits described herein, in partitioning species, in partitioning oligonucleotides, in stimulus-selective release of species from partitions, in performing reactions (e.g., ligation and amplification reactions) in partitions, in performing nucleic acid synthesis reactions, in barcoding nucleic acid, in preparing polynucleotides for sequencing, in sequencing polynucleotides, in mutation detection, in neurologic disorder diagnostics, in diabetes diagnostics, in fetal aneuploidy diagnostics, in cancer mutation detection and forensics, in disease detection, in medical diagnostics, in low input nucleic acid applications, in circulating tumor cell (CTC) sequencing, in polynucleotide phasing, in sequencing polynucleotides from small numbers of cells, in analyzing gene expression, in partitioning polynucleotides from cells, in a combination thereof, and in any other application, method, process or use described herein.

EXAMPLES

Example 1: Production of Capsules by Flow Focusing

Capsules were produced according to the method illustrated in FIG. **5** and the corresponding description of FIG. **5**. The first fluid **501** was an aqueous fluid that contained 8% (w/v) N-isopropylacrylamide, 0.5% (w/v) PLURONIC F67, 2.5% (w/v) ammonium persulfate, and 1% (w/v) N,N'-methylenebisacrylamide. The second fluid **502** was a fluorous oil (HFE-7500) fluid that contained 2% (w/v) KRYTOX FSH and 1% (v/v) N,N,N,N-tetramethylethylene diamine. The T-junction was 100 microns in width, in either direction. The flow rate was adjusted to maintain an oil to aqueous ratio of 2:1 (20 uL/min and 10 uLmin, respectively). The resulting capsules are shown in FIG. **9**A. The shells of the capsules are clearly visible as dark layers surrounding the interior. The size of the capsules is approximately 120 um.

The capsules were washed and resuspended in water. FIG. **9**B shows a micrograph of a single capsule, indicating that the integrity of the capsules is maintained during washing and resuspension.

Example 2: Production of Capsules in Batch

Capsules were produced according to the method illustrated in FIG. **7** and the corresponding description of FIG. **7**. The first capsule precursors **701** contained 8% (w/v) N-isopropylacrylamide, 0.5% (w/v) PLURONIC F67, 2.5% (w/v) ammonium persulfate, and 1% (w/v) N,N'-methylenebisacrylamide stabilized by 2% (w/v) KRYTOX FSL in HFE7500. N,N,N, N-tetramethylethylene diamine was

added to the medium containing the capsule precursors, at a concentration of 2% (w/v). A micrograph of a resulting capsule is shown in FIG. **10**. The shell of the capsules is clearly visible as dark layers surrounding the interior. The size of the capsules is approximately 120 microns.

Example 3: Thermally-Responsive Capsules

Capsules were produced according to the method illustrated in FIG. **7** and the corresponding description of FIG. **7**. The capsule shell wall was produced from N-isopropylacrylamide, a polymer that shrinks at a temperature above 32° C. With reference to FIG. **11**, the capsules were made from a shell comprising the thermally responsive polymer **1101** and contained an aqueous interior **1102**. The capsules were suspended in a oil phase **1103**. Upon raising the temperature above 32° C. (ΔT), the polymer in the capsule shell shrinks, leading to bursting of the capsule and release of the aqueous phase **1102** within the capsule directly into the surrounding oil phase **1103**. The empty capsule shell 1104 is visible.

Example 4: Chemically-Responsive Capsules

Capsules were produced according to the method illustrated in FIG. **7** and the corresponding description of FIG. **7**. The capsule shell wall was produced from a polymer comprising disulfide cross-links, which were dissolved after exposure to dithiothreitol (DTT). FIG. **12** shows selective dissolution of a capsule comprising disulfide cross-links after exposure to a medium containing 0.1M of DTT. The intact capsule **1201** was made with a shell comprising disulfide crosslinks. After 12.5 minutes of exposure to 0.1M DTT, the capsule shell dissolves, releasing the contents of the capsule, as shown in **1202**. The appearance of the capsule at 2.5 minutes and 7.5 minutes is shown in **1203** and **1204**, respectively.

Example 5: Examples of Configurations of Partitions

Many examples of different configurations of partitions are provided throughout this disclosure. FIG. **13** illustrates additional examples of configurations of partitions. In FIG. **13**, the letters "A", "B", and "C" represent polynucleotide barcodes. The letter "S" represents an analyte (e.g., a sample). The letter "Z" represents another species, such as reagents that may be used to attach a barcode to a polynucleotide analyte. These configurations are in no way meant to be limiting and are provided only for the purposes of further illustrating certain embodiments of the invention. As described throughout this disclosure, any suitable configuration of any species (including species that are barcodes, analytes, and reagents) may be used. As described elsewhere in this disclosure, species may be introduced into capsules and droplets using any suitable method. Examples of suitable methods include flow focusing and picoinjection.

The configuration of FIG. **13**A is produced, using the methods described in this disclosure. With reference to FIG. **13**A, an inner capsule or droplet of an emulsion **1301** comprising a barcode (A) is depicted. The inner capsule or droplet of an emulsion **1301** is contained within a partition **1302** that may be an outer capsule or a droplet of an emulsion. The medium between the inner capsule or droplet of an emulsion **1301** and the outer capsule or droplet of an emulsion **1302** comprises an analyte (S) and another species (Z) (e.g., a ligase, polymerase, etc.) for attaching the barcode (A) to the analyte (S). Using the methods described in this disclosure, the inner capsule or droplet of an emulsion **1301** can be induced to release its contents into the outer capsule or droplet of an emulsion **1302** in response to a stimulus, causing mixing of A, S, and Z.

The configuration of FIG. **13**B is produced, using the methods described in this disclosure. With reference to FIG. **13**B, an inner capsule or droplet of an emulsion **1303** comprising two modular components of a barcode (A+B) is depicted. The inner capsule or droplet of an emulsion **1303** is contained within a partition **1304** that may be an outer capsule or droplet of an emulsion. The medium between the inner capsule or droplet of an emulsion **1303** and the outer capsule or droplet of an emulsion **1304** comprises an analyte (S) and another species (Z) (e.g., a ligase, polymerase, etc.) for attaching the barcode (A) to the analyte (S). The other species (Z) may also be used to assemble the two modular components of the barcode (A+B) and add the assembled barcode to the analyte (S), for example in a single ligation step. Using the methods described in this disclosure, the inner capsule or droplet of an emulsion **1303** can be induced to release its contents into the outer capsule or droplet of an emulsion **1304** in response to a stimulus, causing mixing of A, B, S, and Z.

The configuration of FIG. **13**C is produced, using the methods described in this disclosure. With reference to FIG. **13**C, an inner capsule or droplet of an emulsion **1305** comprising a first modular component of a barcode (A) is depicted. The inner capsule or droplet of an emulsion **1305** is contained within a partition **1306** that may be an intermediate capsule or a droplet of an emulsion. The medium between the inner capsule or droplet of an emulsion **1305** and the intermediate capsule or droplet of an emulsion **1306** comprises a second modular component of a barcode (B). The intermediate capsule or droplet of an emulsion **1306** is contained within a partition **1307** that may be an outer capsule or droplet of an emulsion. The medium between the intermediate capsule or droplet of an emulsion **1306** and the outer capsule or droplet of an emulsion **1307** comprises an analyte (S) and another species (Z), each of which may be used as described above. Using the methods described in this disclosure, the inner capsule or droplet of an emulsion **1305** can be induced to release its contents into the intermediate capsule or droplet of an emulsion **1306** in response to a stimulus, causing mixing of A and B. Similarly, the intermediate capsule or droplet of an emulsion **1306** can be induced to release its contents into the outer capsule or droplet of an emulsion **1307**, causing mixing of A, B, S, and Z (if the contents of **1305** have been released) or B, S, and Z (if the contents of **1305** have not been released).

The configuration of FIG. **13**D is produced, using the methods described in this disclosure. With reference to FIG. **13**D, a first inner capsule or droplet of an emulsion **1308** comprises a first modular component of a barcode (A) and a second inner capsule or droplet of an emulsion **1309** comprises a second modular component of a barcode (B). The first and second inner capsules or droplets of an emulsion (**1308** and **1309**) are contained within a partition **1310** that may be an outer capsule or droplet of an emulsion. The medium between the first and second inner capsules or droplets of an emulsion (**1308** and **1309**) and the outer capsule or droplet of an emulsion **1310** comprises an analyte (S) and another species (Z), each of which may be used as described above. Using the methods described in this disclosure, either or both of the first **1308** or second **1309** inner capsules or droplets of an emulsion can be induced to release their contents into the outer capsule or droplet of an emul-

sion **1310** in response to a stimulus, causing mixing of A and/or B (depending on which inner capsules have released their contents), S, and Z.

The configuration of FIG. **13**E is produced, using the methods described in this disclosure. With reference to FIG. **13**E, a first inner capsule or droplet of an emulsion **1311** comprises a first modular component of a barcode (A) and a second inner capsule or droplet of an emulsion **1312** comprises a second modular component of a barcode (B). The first and second inner capsules or droplets of an emulsion (**1311** and **1312**) are contained within an intermediate capsule or droplet of an emulsion **1313**. The medium between the first and second inner capsules or droplets of an emulsion (**1311** and **1312**) and the intermediate capsule or droplet of an emulsion **1313** comprises a third modular component of a barcode (C). The intermediate capsule or droplet of an emulsion **1313** is contained within an outer capsule or droplet of an emulsion **1314**. The medium between the intermediate capsule or droplet of an emulsion **1313** and the outer capsule or droplet of an emulsion **1314** comprises an analyte (S) and another species (Z), each of which may be used as described above. Using the methods described in this disclosure, either or both of the first **1311** or second **1312** inner capsules or droplets of an emulsion can be induced to release their contents into the intermediate capsule or droplet of an emulsion **1313** in response to a stimulus, causing mixing of A and/or B (depending on which inner capsules have released their contents) with C. Similarly, the intermediate capsule or droplet of an emulsion **1313** can be induced to release its contents into the outer capsule or droplet of an emulsion **1314**, causing mixing of A, B, C, S, and Z, depending on which contents of **1311** and **1312** have been released.

Example 6: Spotting of Species within Wells

A well comprising spots in the configuration of FIG. **14** is produced, using the methods described in this disclosure. With reference to FIG. **14**, the bottom surface of a well **1401** is depicted. The shapes used for the well and the spots are merely for illustrative purposes and in no way meant to be limiting. Any suitable shape may be used for the well and/or any spot. With reference to FIG. **14**, four spots are shown, each spot in one quadrant of the bottom surface of the well. The number of spots, and the contents of the spots, are also merely illustrative. Any number of spots or suitable contents of spots may be used. In FIG. **14**, spot **1402** comprises a first modular component of a barcode (A), spot **1403** comprises a second modular component of a barcode (B), spot **1404** comprises a third modular component of a barcode (C), and spot **1405** comprises a reagent (Z). The spots are separated, to prevent mixing of the contents of the spots before a sample is added. By adding a sample (e.g., an analyte in a medium) to the well, the contents of the spots can be mixed with the sample at the appropriate time.

Example 7: Sonication of Encapsulated Polynucleotide

Polynucleotides (e.g., genomic DNA) are isolated from cells according to methods known in the art. The polynucleotides are encapsulated in a capsule and/or within a hydrogel matrix. The polynucleotides are fragmented by exposing the capsules and/or hydrogel matrix to shear stress induced by ultrasonic waves. Sheared, encapsulated polynucleotide is generated.

With reference to FIG. **15**A, a polynucleotide (e.g., isolated from a cell) is encapsulated in a capsule or a gel droplet **1501**. As described elsewhere in this disclosure, the polynucleotide can be encapsulated such that each capsule or gel droplet comprises only a single copy of a particular polynucleotide, so that each capsule or gel droplet contains a mixture of non-overlapping fragments. The capsule or gel droplet is sonicated **1502** to shear the polynucleotide. The capsule or gel droplet is configured to withstand the sonication. The result is a capsule or gel droplet comprising fragmented polynucleotide **1503**.

The encapsulated fragmented polynucleotide can then be processed according to any suitable method, including methods known in the art and methods described in this disclosure. FIG. **15**B shows an example of further processing. With reference to FIG. **15**B, the a first inner capsule or hydrogel droplet comprising sheared polynucleotides **1504** (as generated, e.g., in FIG. **15**A) is encapsulated into an outer capsule **1506**. The outer capsule **1506** also comprises a second inner capsule **1505**. The second inner capsule **1505** comprises two modular components of a barcode (A+B). The medium between the outer capsule **1506** and the two inner capsules **1504** and **1505** comprises a reagent (Z). Upon releasing the contents of the inner capsules, the sheared polynucleotide will mix with A, B, and Z. In some cases, a plurality of capsules or hydrogel droplets comprising a sheared polynucleotide (i.e., a plurality of **1504**s) may be encapsulated in the outer capsule **1506**.

While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

What is claimed is:

1. A composition comprising a plurality of capsules, said capsules situated within droplets in an emulsion, wherein said capsules are configured to release their contents into said droplets upon the application of a stimulus to provide said contents in said droplets in said emulsion, wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof.

2. The composition of claim **1**, wherein at least one of said capsules and said droplets comprise a species selected from the group consisting of a reagent and an analyte.

3. The composition of claim **2**, wherein said reagent is selected from the group consisting of a protein, a polynucleotide, an enzyme, an antibody, a barcode, an adapter, a buffer, a small molecule, a detergent, a dye, a polymer and combinations thereof.

4. The composition of claim **3**, wherein said enzyme is selected from the group consisting of a proteinase, a restriction enzyme, a ligase, a polymerase, a fragmentase, a reverse transcriptase, a transposase, and combinations thereof.

5. The composition of claim **4**, wherein said restriction enzyme is a restriction enzyme that is a rare cutter.

6. The composition of claim **3**, wherein said barcode is an oligonucleotide barcode.

7. The composition of claim **2**, wherein said analyte is selected from the group consisting of a cell, a polynucle-

otide, a chromosome, a protein, a peptide, a polysaccharide, a sugar, a lipid, a small molecule, and combinations thereof.

8. The composition of claim **7**, wherein said analyte is a polynucleotide.

9. The composition of claim **8**, wherein said polynucleotide is selected from the group consisting of DNA, RNA, cDNA, and combinations thereof.

10. The composition of claim **8**, wherein the amount of said polynucleotide in said composition is an amount sufficient to provide about 100-200X sequence coverage.

11. The composition of claim **7**, wherein each capsule comprises, on average, about one chromosome.

12. The composition of claim **1**, wherein each droplet comprises, on average, about 1 capsule per droplet.

13. The composition of claim **1**, wherein each droplet can hold, at most, a single capsule.

14. The composition of claim **1**, wherein at least one of said capsules comprises a further partition.

15. The composition of claim **14**, wherein said further partition is selected from the group consisting of a capsule and a droplet in an emulsion.

16. The composition of claim **1**, wherein at least one of said capsules has a shell selected from the group consisting of a polymeric shell, a hydrogel, a hydrophilic shell, a hydrophobic shell, a shell with a net positive charge, a shell with a net negative charge, a shell with a neutral charge, and combinations thereof.

17. The composition of claim **1**, wherein said droplets comprise a fluid that is of a lesser density than the density of said capsules.

18. The composition of claim **1**, wherein said droplets comprise a fluid that is of a greater density than the density of said capsules.

19. The composition of claim **1**, wherein said capsules are produced by a method selected from the group consisting of emulsification polymerization, layer-by-layer assembly with polyelectrolytes, coacervation, internal phase separation, flow focusing, and combinations thereof.

20. The composition of claim **1**, wherein said stimulus is applied to said capsules.

21. The composition of claim **1**, wherein said stimulus is applied to said droplets.

22. A composition comprising a plurality of outer capsules in an emulsion, said outer capsules comprising at least one inner capsule, wherein said at least one inner capsule is configured to release its contents into at least one outer capsule among said plurality of outer capsules upon the application of a stimulus to provide said contents in said outer capsule in said emulsion, wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof.

23. A device comprising a plurality of partitions, wherein at least one partition of said plurality of partitions comprises a capsule, wherein said capsule is situated within a droplet in an emulsion, wherein said capsule is configured to release its contents into said droplet upon the application of a stimulus to provide said contents in said droplet in said emulsion, wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof.

24. A device comprising a plurality of partitions, wherein at least one partition of said plurality of partitions comprises an outer capsule in an emulsion, said outer capsule comprising at least one inner capsule, wherein said at least one inner capsule is configured to release its contents into said outer capsule upon the application of a stimulus to provide said contents in said outer capsule in said emulsion, wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof.

25. A method comprising:

a. providing a plurality of capsules, said capsules situated within droplets in an emulsion, wherein said capsules are configured to release their contents into said droplets upon the application of a stimulus, wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof; and

b. providing a stimulus to cause said capsules to release their contents into said droplets in said emulsion.

26. A method comprising:

a. providing a plurality of inner capsules, said inner capsules situated within outer capsules in an emulsion, wherein said inner capsules are configured to release their contents into said outer capsules upon the application of a stimulus, wherein said stimulus is selected from the group consisting of a change in pH, a change in ion concentration, reduction of disulfide bonds, and combinations thereof; and

b. providing a stimulus to cause said inner capsules to release their contents into said outer capsules in said emulsion.

27. The composition of claim **1**, wherein said contents comprise at least 10,000 barcoded oligonucleotides releasably attached to each of said capsules.

28. The composition of claim **22**, wherein said contents comprise at least 10,000 barcoded oligonucleotides releasably attached to said inner capsule.

29. The device of claim **23**, wherein said contents comprise at least 10,000 barcoded oligonucleotides releasably attached to said capsule.

30. The device of claim **24**, wherein said contents comprise at least 10,000 barcoded oligonucleotides releasably attached to said inner capsule.

31. The method of claim **25**, wherein said contents comprise at least 10,000 barcoded oligonucleotides releasably attached to each of said capsule.

32. The method of claim **26**, wherein said contents comprise at least 10,000 barcoded oligonucleotides releasably attached to each of said inner capsule.

33. The composition of claim **1**, wherein said capsules are gels.

34. The composition of claim **22**, wherein said inner capsule is a gel.

35. The composition of claim **22**, wherein at least one of said inner capsules and said outer capsules comprise a species selected from the group consisting of a reagent and an analyte.

36. The composition of claim **35**, wherein said reagent is selected from the group consisting of a protein, a polynucleotide, an enzyme, an antibody, a barcode, an adaptor, a buffer, a small molecule, a detergent, a dye, a polymer and combinations thereof.

37. The composition of claim **36**, wherein said enzyme is selected from the group consisting of a proteinase, a restriction enzyme, a ligase, a polymerase, a fragmentase, a reverse transcriptase, a transposase, and combinations thereof.

38. The composition of claim **36**, wherein said barcode is an oligonucleotide barcode.

39. The composition of claim **35**, wherein said analyte is selected from the group consisting of a cell, a polynucleotide, a chromosome, a protein, a peptide, a polysaccharide, a sugar, a lipid, a small molecule, and combinations thereof.

40. The composition of claim **39**, wherein said analyte is a polynucleotide.

* * * * *

JX-0003

US009689024B2

(12) **United States Patent**
Hindson et al.

(10) **Patent No.: US 9,689,024 B2**
(45) **Date of Patent: *Jun. 27, 2017**

(54) **METHODS FOR DROPLET-BASED SAMPLE PREPARATION**

(71) Applicant: **10X Genomics, Inc.**, Pleasanton, CA (US)

(72) Inventors: **Benjamin Hindson**, Pleasanton, CA (US); **Serge Saxonov**, Oakland, CA (US); **Michael Schnall-Levin**, San Francisco, CA (US)

(73) Assignee: **10X GENOMICS, INC.**, Pleasanton, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **14/624,468**

(22) Filed: **Feb. 17, 2015**

(65) **Prior Publication Data**

US 2015/0225777 A1 Aug. 13, 2015

Related U.S. Application Data

(62) Division of application No. 13/966,150, filed on Aug. 13, 2013.

(60) Provisional application No. 61/683,192, filed on Aug. 14, 2012, provisional application No. 61/737,374, filed on Dec. 14, 2012, provisional application No. 61/762,435, filed on Feb. 8, 2013, provisional application No. 61/800,223, filed on Mar. 15, 2013, provisional application No. 61/840,403, filed on Jun. 27, 2013, provisional application No. 61/844,804, filed on Jul. 10, 2013.

(51) **Int. Cl.**

C12Q 1/68	(2006.01)
C12P 19/34	(2006.01)
C07H 21/04	(2006.01)
G01N 15/06	(2006.01)
C40B 40/06	(2006.01)
C40B 50/14	(2006.01)
C12N 15/10	(2006.01)
B01L 3/00	(2006.01)
B01J 19/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C12Q 1/6806*** (2013.01); ***B01J 19/0046*** (2013.01); ***B01L 3/508*** (2013.01); ***B01L 3/502715*** (2013.01); ***B01L 3/523*** (2013.01); ***C12N 15/1065*** (2013.01); *B01L 2200/0647* (2013.01); *B01L 2400/0677* (2013.01)

(58) **Field of Classification Search**

CPC . C12Q 1/68; C12M 1/00; G01N 33/48; B01L 3/00

USPC 435/6.1, 91.1, 91.2, 287.2; 536/24.3, 536/24.33; 422/68.1; 506/16, 30, 31

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,582,802	A	4/1986	Zimmerman et al.
5,149,625	A	9/1992	Church et al.
5,202,231	A	4/1993	Drmanac et al.
5,413,924	A	5/1995	Kozak et al.
5,436,130	A	7/1995	Mathies et al.
5,512,131	A	4/1996	Kumar et al.
5,587,128	A	12/1996	Wilding et al.
5,605,793	A	2/1997	Stemmer
5,618,711	A	4/1997	Gelfand et al.
5,695,940	A	12/1997	Drmanac et al.
5,736,330	A	4/1998	Fulton
5,756,334	A	5/1998	Perler et al.
5,834,197	A	11/1998	Parton
5,851,769	A	12/1998	Gray et al.
5,856,174	A	1/1999	Lipshutz et al.
5,958,703	A	9/1999	Dower et al.
5,994,056	A	11/1999	Higuchi et al.
6,046,003	A	4/2000	Mandecki
6,051,377	A	4/2000	Mandecki
6,057,107	A	5/2000	Fulton
6,103,537	A	8/2000	Ullman et al.
6,143,496	A	11/2000	Brown et al.
6,172,218	B1	1/2001	Brenner
6,265,552	B1	7/2001	Schatz

(Continued)

FOREIGN PATENT DOCUMENTS

EP	0249007 A2	12/1987
EP	0271281 A2	6/1988
EP	0637996 B1	7/1997
EP	1019496 B1	9/2004
EP	1482036 B1	10/2007
EP	1594980 B1	11/2009
EP	1967592 B1	4/2010
EP	2258846 A2	12/2010
EP	2145955 B1	2/2012
EP	1905828 B1	8/2012
EP	2136786 B1	10/2012
EP	1908832 B1	12/2012
EP	2540389 A1	1/2013

(Continued)

OTHER PUBLICATIONS

Esser-Kahn et al, Triggered Release from Polymer Capsules, 2011, Macromolecules, 44, 5539-5553.*

(Continued)

Primary Examiner — Narayan Bhat

(74) *Attorney, Agent, or Firm* — Wilson Sonsini Goodrich & Rosati

(57) **ABSTRACT**

This disclosure provides microwell capsule array devices. The microwell capsule array devices are generally capable of performing one or more sample preparation operations. Such sample preparation operations may be used as a prelude to one more or more analysis operations. For example, a device of this disclosure can achieve physical partitioning and discrete mixing of samples with unique molecular identifiers within a single unit in preparation for various analysis operations. The device may be useful in a variety of applications and most notably nucleic-acid-based sequencing, detection and quantification of gene expression and single-cell analysis.

22 Claims, 4 Drawing Sheets

10XG-0000054790

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,297,006 B1 10/2001 Drmanac et al.
6,297,017 B1 10/2001 Schmidt et al.
6,327,410 B1 12/2001 Walt et al.
6,355,198 B1 3/2002 Kim et al.
6,361,950 B1 3/2002 Mandecki
6,372,813 B1 4/2002 Johnson et al.
6,406,848 B1 6/2002 Bridgham et al.
6,432,360 B1 8/2002 Church
6,485,944 B1 11/2002 Church et al.
6,511,803 B1 1/2003 Church et al.
6,524,456 B1 2/2003 Ramsey et al.
6,586,176 B1 7/2003 Trnovsky et al.
6,632,606 B1 10/2003 Ullman et al.
6,632,655 B1 10/2003 Mehta et al.
6,670,133 B2 12/2003 Knapp et al.
6,767,731 B2 7/2004 Hannah
6,800,298 B1 10/2004 Burdick et al.
6,806,052 B2 10/2004 Bridgham et al.
6,806,058 B2 10/2004 Jesperson et al.
6,859,570 B2 2/2005 Walt et al.
6,884,788 B2 4/2005 Bulpitt et al.
6,913,935 B1 7/2005 Thomas
6,929,859 B2 8/2005 Chandler et al.
6,969,488 B2 11/2005 Bridgham et al.
6,974,669 B2 12/2005 Mirkin et al.
7,041,481 B2 5/2006 Anderson et al.
7,115,400 B1 10/2006 Adessi et al.
7,129,091 B2 10/2006 Ismagilov et al.
7,268,167 B2 9/2007 Higuchi et al.
7,282,370 B2 10/2007 Bridgham et al.
7,297,485 B2 11/2007 Bornarth et al.
7,323,305 B2 1/2008 Leamon et al.
7,425,431 B2 9/2008 Church et al.
7,536,928 B2 5/2009 Kazuno
7,604,938 B2 10/2009 Takahashi et al.
7,622,280 B2 11/2009 Holliger et al.
7,638,276 B2 12/2009 Griffiths et al.
7,645,596 B2 1/2010 Williams et al.
7,666,664 B2 2/2010 Sarofim et al.
7,708,949 B2 5/2010 Stone et al.
7,709,197 B2 5/2010 Drmanac
7,745,178 B2 6/2010 Dong et al.
7,776,927 B2 8/2010 Chu et al.
RE41,780 E 9/2010 Anderson et al.
7,799,553 B2 9/2010 Mathies et al.
7,842,457 B2 11/2010 Berka et al.
7,901,891 B2 3/2011 Drmanac et al.
7,910,354 B2 3/2011 Drmanac et al.
7,960,104 B2 6/2011 Drmanac et al.
7,968,287 B2 6/2011 Griffiths et al.
7,972,778 B2 7/2011 Brown et al.
8,003,312 B2 8/2011 Krutzik et al.
8,053,192 B2 11/2011 Bignell et al.
8,067,159 B2 11/2011 Brown et al.
8,133,719 B2 3/2012 Drmanac et al.
8,252,539 B2 8/2012 Quake et al.
8,268,564 B2 9/2012 Roth et al.
8,273,573 B2 9/2012 Ismagilov et al.
8,278,071 B2 10/2012 Brown et al.
8,304,193 B2 11/2012 Ismagilov et al.
8,329,407 B2 12/2012 Ismagilov et al.
8,337,778 B2 12/2012 Stone et al.
8,592,150 B2 11/2013 Drmanac et al.
8,603,749 B2 12/2013 Gillevet
8,679,756 B1 3/2014 Brenner et al.
8,748,094 B2 6/2014 Weitz et al.
8,748,102 B2 6/2014 Berka et al.
8,765,380 B2 7/2014 Berka et al.
8,822,148 B2 9/2014 Ismagliov et al.
8,835,358 B2 9/2014 Fodor et al.
8,871,444 B2 10/2014 Griffiths et al.
8,889,083 B2 11/2014 Ismagilov et al.
8,986,286 B2 3/2015 Tanghoej et al.
9,012,390 B2 4/2015 Holtze et al.
9,017,948 B2 4/2015 Agresti et al.
9,029,083 B2 5/2015 Griffiths et al.
9,249,460 B2 2/2016 Pushkarev et al.
9,347,059 B2 5/2016 Saxonov
9,388,465 B2 7/2016 Hindson et al.
9,410,201 B2 8/2016 Hindson et al.
9,567,631 B2 2/2017 Hindson et al.
2001/0020588 A1 9/2001 Adourian et al.
2001/0044109 A1 11/2001 Mandecki
2002/0034737 A1 3/2002 Drmanac
2002/0051992 A1 5/2002 Bridgham et al.
2002/0089100 A1 7/2002 Kawasaki
2002/0092767 A1 7/2002 Bjornson et al.
2002/0179849 A1 12/2002 Maher et al.
2003/0008285 A1 1/2003 Fischer
2003/0008323 A1 1/2003 Ravkin et al.
2003/0027221 A1 2/2003 Scott et al.
2003/0028981 A1 2/2003 Chandler et al.
2003/0039978 A1 2/2003 Hannah
2003/0044777 A1 3/2003 Beattie
2003/0044836 A1 3/2003 Levine et al.
2003/0082587 A1 5/2003 Seul et al.
2003/0104466 A1 6/2003 Knapp et al.
2003/0108897 A1 6/2003 Drmanac
2003/0149307 A1 8/2003 Hai et al.
2003/0170698 A1 9/2003 Gascoyne et al.
2003/0182068 A1 9/2003 Battersby et al.
2003/0207260 A1 11/2003 Trnovsky et al.
2003/0215862 A1 11/2003 Parce et al.
2004/0063138 A1 4/2004 McGinnis et al.
2004/0132122 A1 7/2004 Banerjee et al.
2004/0258701 A1 12/2004 Dominowski et al.
2005/0019839 A1 1/2005 Jespersen et al.
2005/0042625 A1 2/2005 Schmidt et al.
2005/0079510 A1 4/2005 Berka et al.
2005/0130188 A1 6/2005 Walt et al.
2005/0172476 A1 8/2005 Stone et al.
2005/0181379 A1 8/2005 Su et al.
2005/0202429 A1 9/2005 Trau et al.
2005/0202489 A1 9/2005 Cho et al.
2005/0221339 A1 10/2005 Griffiths et al.
2005/0244850 A1 11/2005 Huang et al.
2005/0287572 A1 12/2005 Mathies et al.
2006/0002890 A1 1/2006 Hersel et al.
2006/0020371 A1 1/2006 Ham et al.
2006/0073487 A1 4/2006 Oliver et al.
2006/0078888 A1 4/2006 Griffiths et al.
2006/0153924 A1 7/2006 Griffiths et al.
2006/0163385 A1 7/2006 Link et al.
2006/0199193 A1 9/2006 Koo et al.
2006/0240506 A1 10/2006 Kushmaro et al.
2006/0257893 A1 11/2006 Takahashi et al.
2006/0263888 A1 11/2006 Fritz et al.
2006/0292583 A1 12/2006 Schneider et al.
2007/0003442 A1 1/2007 Link et al.
2007/0020617 A1 1/2007 Trnovsky et al.
2007/0054119 A1 3/2007 Garstecki et al.
2007/0077572 A1 4/2007 Tawfik et al.
2007/0092914 A1 4/2007 Griffiths et al.
2007/0099208 A1 5/2007 Drmanac et al.
2007/0154903 A1 7/2007 Marla et al.
2007/0160503 A1 7/2007 Sethu et al.
2007/0172873 A1 7/2007 Brenner et al.
2007/0190543 A1 8/2007 Livak
2007/0195127 A1 8/2007 Ahn et al.
2007/0207060 A1 9/2007 Zou et al.
2007/0228588 A1 10/2007 Noritomi et al.
2007/0264320 A1 11/2007 Lee et al.
2008/0003142 A1 1/2008 Link et al.
2008/0004436 A1 1/2008 Tawfik et al.
2008/0014589 A1 1/2008 Link et al.
2008/0213766 A1 9/2008 Brown et al.
2008/0228268 A1 9/2008 Shannon et al.
2008/0241820 A1 10/2008 Krutzik et al.
2009/0005252 A1 1/2009 Drmanac et al.
2009/0011943 A1 1/2009 Drmanac et al.
2009/0012187 A1 1/2009 Chu et al.
2009/0025277 A1 1/2009 Takanashi
2009/0035770 A1 2/2009 Mathies et al.
2009/0048124 A1 2/2009 Leamon et al.

(56) **References Cited**

U.S. PATENT DOCUMENTS

2009/0053169	A1	2/2009	Castillo et al.
2009/0068170	A1	3/2009	Weitz et al.
2009/0098555	A1	4/2009	Roth et al.
2009/0118488	A1	5/2009	Drmanac et al.
2009/0137404	A1	5/2009	Drmanac et al.
2009/0137414	A1	5/2009	Drmanac et al.
2009/0143244	A1	6/2009	Bridgham et al.
2009/0148961	A1	6/2009	Luchini et al.
2009/0155781	A1	6/2009	Drmanac et al.
2009/0197248	A1	8/2009	Griffiths et al.
2009/0197772	A1	8/2009	Griffiths et al.
2009/0202984	A1	8/2009	Cantor
2009/0203531	A1	8/2009	Kurn
2009/0264299	A1	10/2009	Drmanac et al.
2009/0286687	A1	11/2009	Dressman et al.
2010/0021973	A1	1/2010	Makarov et al.
2010/0021984	A1	1/2010	Edd et al.
2010/0022414	A1	1/2010	Link et al.
2010/0035254	A1	2/2010	Williams
2010/0062494	A1	3/2010	Church et al.
2010/0069263	A1	3/2010	Shendure et al.
2010/0105112	A1	4/2010	Holtze et al.
2010/0130369	A1	5/2010	Shenderov et al.
2010/0136544	A1	6/2010	Agresti et al.
2010/0137163	A1	6/2010	Link et al.
2010/0173394	A1	7/2010	Colston, Jr. et al.
2010/0210479	A1	8/2010	Griffiths et al.
2011/0033548	A1	2/2011	Lai et al.
2011/0033854	A1	2/2011	Drmanac et al.
2011/0053798	A1	3/2011	Hindson et al.
2011/0071053	A1	3/2011	Drmanac et al.
2011/0086780	A1	4/2011	Colston, Jr. et al.
2011/0092376	A1	4/2011	Colston, Jr. et al.
2011/0092392	A1	4/2011	Colston, Jr. et al.
2011/0160078	A1	6/2011	Fodor et al.
2011/0195496	A1	8/2011	Muraguchi et al.
2011/0201526	A1	8/2011	Berka et al.
2011/0217736	A1	9/2011	Hindson
2011/0218123	A1	9/2011	Weitz et al.
2011/0263457	A1	10/2011	Krutzik et al.
2011/0267457	A1	11/2011	Weitz et al.
2011/0281738	A1	11/2011	Drmanac et al.
2011/0305761	A1	12/2011	Shum et al.
2011/0319281	A1	12/2011	Drmanac
2012/0000777	A1	1/2012	Garrell et al.
2012/0010098	A1	1/2012	Griffiths et al.
2012/0010107	A1	1/2012	Griffiths et al.
2012/0015382	A1	1/2012	Weitz et al.
2012/0015822	A1	1/2012	Weitz et al.
2012/0071331	A1	3/2012	Casbon et al.
2012/0121481	A1	5/2012	Romanowsky et al.
2012/0132288	A1	5/2012	Weitz et al.
2012/0135893	A1	5/2012	Drmanac et al.
2012/0172259	A1	7/2012	Rigatti et al.
2012/0190032	A1	7/2012	Ness et al.
2012/0196288	A1	8/2012	Beer
2012/0211084	A1	8/2012	Weitz et al.
2012/0220494	A1	8/2012	Samuels et al.
2012/0220497	A1	8/2012	Jacobson et al.
2012/0222748	A1	9/2012	Weitz et al.
2012/0309002	A1	12/2012	Link
2012/0316074	A1	12/2012	Saxonov
2013/0018970	A1	1/2013	Woundy et al.
2013/0028812	A1	1/2013	Prieto et al.
2013/0046030	A1	2/2013	Rotem et al.
2013/0078638	A1	3/2013	Berka et al.
2013/0079231	A1	3/2013	Pushkarev et al.
2013/0109575	A1	5/2013	Kleinschmidt et al.
2013/0157870	A1	6/2013	Pushkarev et al.
2013/0157899	A1	6/2013	Adler, Jr. et al.
2013/0178368	A1	7/2013	Griffiths et al.
2013/0189700	A1	7/2013	So et al.
2013/0203675	A1	8/2013	Desimone et al.
2013/0210639	A1	8/2013	Link et al.
2013/0210991	A1	8/2013	Fonnum et al.
2013/0211055	A1	8/2013	Raines et al.
2013/0225418	A1	8/2013	Watson
2013/0274117	A1	10/2013	Church et al.
2013/0296173	A1	11/2013	Callow et al.
2013/0343317	A1	12/2013	Etemad et al.
2014/0037514	A1	2/2014	Stone et al.
2014/0057799	A1	2/2014	Johnson et al.
2014/0065234	A1	3/2014	Shum et al.
2014/0155295	A1	6/2014	Hindson et al.
2014/0194323	A1	7/2014	Gillevet
2014/0199730	A1	7/2014	Agresti et al.
2014/0199731	A1	7/2014	Agresti et al.
2014/0206554	A1	7/2014	Hindson et al.
2014/0227684	A1	8/2014	Hindson et al.
2014/0227706	A1	8/2014	Kato et al.
2014/0228255	A1	8/2014	Hindson et al.
2014/0235506	A1	8/2014	Hindson et al.
2014/0287963	A1	9/2014	Hindson et al.
2014/0302503	A1	10/2014	Lowe et al.
2014/0378322	A1	12/2014	Hindson et al.
2014/0378345	A1	12/2014	Hindson et al.
2014/0378349	A1	12/2014	Hindson et al.
2014/0378350	A1	12/2014	Hindson et al.
2015/0005199	A1	1/2015	Hindson et al.
2015/0005200	A1	1/2015	Hindson et al.
2015/0011430	A1	1/2015	Saxonov
2015/0011432	A1	1/2015	Saxonov
2015/0111256	A1	4/2015	Church et al.
2015/0119280	A1	4/2015	Srinivas et al.
2015/0218633	A1	8/2015	Hindson et al.
2015/0224466	A1	8/2015	Hindson et al.
2015/0225778	A1	8/2015	Hindson et al.
2015/0292988	A1	10/2015	Bharadwaj et al.
2015/0298091	A1	10/2015	Weitz et al.
2015/0299772	A1	10/2015	Zhang
2015/0376605	A1	12/2015	Jarosz et al.
2015/0376609	A1	12/2015	Hindson et al.
2015/0376700	A1	12/2015	Schnall-Levin et al.
2015/0379196	A1	12/2015	Schnall-Levin et al.
2016/0024558	A1	1/2016	Hardenbol et al.
2016/0122817	A1	5/2016	Jarosz et al.
2016/0244742	A1	8/2016	Linnarsson et al.
2016/0257984	A1	9/2016	Hardenbol et al.
2016/0304860	A1	10/2016	Hindson et al.
2016/0348093	A1	12/2016	Price et al.
2017/0016041	A1	1/2017	Greenfield et al.

FOREIGN PATENT DOCUMENTS

EP	2752664	A1	7/2014
GB	2485850	A	5/2012
JP	S 5949832	A	3/1984
JP	S60227826	A	11/1985
JP	2006-507921	A	3/2006
JP	2006-289250	A	10/2006
JP	2007015990	A	1/2007
JP	2007-268350	A	10/2007
JP	2009-208074	A	9/2009
WO	WO-9530782	A1	11/1995
WO	WO 96/29629	A2	3/1996
WO	WO 96/41011	A1	12/1996
WO	WO 99/09217	A1	2/1999
WO	WO 99/52708	A1	10/1999
WO	WO 00/08212	A1	2/2000
WO	WO 00/26412	A1	5/2000
WO	WO-0070095	A2	11/2000
WO	WO 01/14589	A2	3/2001
WO	WO 01/89787	A2	11/2001
WO	WO 02/31203	A2	4/2002
WO	WO 02/086148	A1	10/2002
WO	WO 2004/002627	A2	1/2004
WO	WO 2004/010106	A2	1/2004
WO	WO-2004069849	A2	8/2004
WO	WO 2004/091763	A2	10/2004
WO	WO 2005/040406	A1	10/2004
WO	WO 2004/102204	A1	11/2004
WO	WO 2004/103565	A2	12/2004
WO	WO 2004/105734	A1	12/2004
WO	WO-2005002730	A1	1/2005

(56) **References Cited**

FOREIGN PATENT DOCUMENTS

WO	WO 2005/082098	A2	2/2005
WO	WO 2005/021151	A1	3/2005
WO	WO 2005/023331	A2	3/2005
WO	WO 2005/049787	A2	6/2005
WO	WO 2006/030993	A1	3/2006
WO	WO 2006/078841	A1	7/2006
WO	WO 2006/096571	A2	9/2006
WO	WO 2007/001448	A2	1/2007
WO	WO 2007/002490	A2	1/2007
WO	WO 2007/024840	A2	3/2007
WO	WO 2007/081385	A2	7/2007
WO	WO 2007/081387	A1	7/2007
WO	WO 2007/089541	A2	8/2007
WO	WO 2008/021123	A1	8/2007
WO	WO 2007/114794	A1	10/2007
WO	WO 2007/121489	A2	10/2007
WO	WO 2007/133710	A2	11/2007
WO	WO 2007/138178	A2	12/2007
WO	WO 2007/139766	A2	12/2007
WO	WO 2007/140015	A2	12/2007
WO	WO 2007/149432	A2	12/2007
WO	WO 2008/091792	A2	1/2008
WO	WO 2008/102057	A1	8/2008
WO	WO 2008/109176	A2	9/2008
WO	WO 2008/121342	A2	10/2008
WO	WO 2008/134153	A1	11/2008
WO	WO 2009/005680	A1	1/2009
WO	WO 2009/011808	A1	1/2009
WO	WO 2009/061372	A1	5/2009
WO	WO 2009/085215	A1	7/2009
WO	WO 2010/004018	A2	1/2010
WO	WO 2010/033200	A2	3/2010
WO	WO-2010104604	A1	9/2010
WO	WO-2010115154	A1	10/2010
WO	WO 2010/148039	A2	12/2010
WO	WO 2010/151776	A2	12/2010
WO	WO 2011/047870	A1	4/2011
WO	WO 2011/056546	A1	5/2011
WO	WO 2011/066476	A1	6/2011
WO	WO-2011074960	A1	6/2011
WO	WO 2012/012037	A1	1/2012
WO	WO 2012/048341	A1	4/2012
WO	WO 2012/083225	A2	6/2012
WO	WO-2012106546	A2	8/2012
WO	WO-2012112970	A2	8/2012
WO	WO-2012142611	A2	10/2012
WO	WO 2012/149042	A2	11/2012
WO	WO 2012/166425	A2	12/2012
WO	WO-2013019751	A1	2/2013
WO	WO-2013036929	A1	3/2013
WO	WO-2013122996	A1	8/2013
WO	WO-2013123125	A1	8/2013
WO	WO 2013/177220	A1	11/2013
WO	WO 2014/028537	A1	2/2014
WO	WO-2014074611	A1	5/2014
WO	WO-2014093676	A1	6/2014
WO	WO-2014210353	A2	12/2014
WO	WO-2015044428	A1	4/2015
WO	WO-2015164212	A1	10/2015

OTHER PUBLICATIONS

U.S. Appl. No. 14/680,808, filed Apr. 7, 2015, Hindson et al.
International search report and written opinion dated May 14, 2015 for PCT/US2014/044398.
Office action dated Sep. 16, 2015 for U.S. Appl. No. 14/175,973.
Office action dated Sep. 25, 2015 for U.S. Appl. No. 14/250,701.
U.S. Appl. No. 14/682,952, filed Apr. 8, 2015, Bharadwaj et al.
International search report and written opinion dated Aug. 19, 2015 for PCT/US2015/025197.
Office action dated Oct. 9, 2015 for U.S. Appl. No. 14/680,808.
Office action dated Nov. 6, 2015 for U.S. Appl. No. 13/966,150.
U.S. Appl. No. 14/624,473, filed Feb. 17, 2015, Hindson et al.
U.S. Appl. No. 14/624,484, filed Feb. 17, 2015, Hindson et al.
Abate et al., Valve-based flow focusing for drog formation. Appl Phys Left. 2009;94. 3 pages.
Abate, et al. High-throughput injection with microfluidics using picoinjectors. Proc Natl Acad Sci U S A. Nov. 9, 2010;107(45):19163-6. doi: 10.1073/pNas.1006888107. Epub Oct. 20, 2010.
Advisory Action dated Mar. 21, 2014 for U.S. Appl. No. 13/119,470.
Advisory Action dated May 16, 2014 for U.S. Appl. No. 13/503,588.
Advisory Action mailed Nov. 20, 2013 for U.S. Appl. No. 13/139,326.
Agresti, et al. Selection of ribozymes that catalyse multiple-turnover Diels-Alder cycloadditions by using in vitro compartmentalization. Proc Natl Acad Sci U S A. Nov. 8, 2005;102(45):16170-5. Epub Oct. 31, 2005.
Akselband, "Enrichment of slow-growing marine microorganisms from mixed cultures using gel microdrop (GMD) growth assay and fluorescence-activated cell sorting", J. Exp. Marine Bioi., 329: 196-205 (2006).
Akselband, "Rapid mycobacteria drug susceptibility testing using gel microdrop (GMD) growth assay and flow cytometry", J. Microbiol. Methods, 62:181-197 (2005).
Anna et al., "Formation of dispersions using 'flow focusing' in microchannels", Appln. Phys. Letts. 82:3 364 (2003).
Australian Office Action issued Dec. 17, 2013 for Application No. AU 2010315580.
Boone, et al. Plastic advances microfluidic devices. The devices debuted in silicon and glass, but plastic fabrication may make them hugely successful in biotechnology application. Analytical Chemistry. Feb. 2002; 78A-86A.
Braeckmans et al., Scanning the Code. Modern Drug Discovery. 2003:28-32.
Bransky, et al. A microfluidic droplet generator based on a piezoelectric actuator. Lab Chip. Feb. 21, 2009;9(4):516-20. doi: 10.1039/b814810d. Epub Nov. 20, 2008.
Carroll, "The selection of high-producing cell lines using flow cytometry and cell sorting", Exp. Op. Bioi. Therp., 4:11 1821-1829 (2004).
Chaudhary "A rapid method of cloning functioNal variable-region antibody genese in *Escherichia coli* as single-chain immunotoxins" Proc. Nat!. Acad. Sci USA 87: 1066-1070 (Feb. 1990).
Chechetkin et al., Sequencing by hybridization with the generic 6-mer oligonucleotide microarray: an advanced scheme for data processing. J Biomol Struct Dyn. Aug. 2000;18(1):83-101.
Chinese Office Action and search report mailed May 23, 2013 for Application No. CN 200880127116.4.
Chinese office action dated Jun. 18, 2012 for CN Application No. 200880127116.4.
Chou, et al. Disposable Microdevices for DNA Analysis and Cell Sorting. Proc. Solid-State Sensor and Actuator Workshop, Hilton Head, SC. Jun. 8-11, 1998; 11-14.
Clausell-Tormos et al., "Droplet-based microfluidic platforms for the encapsulation and screening of mammalian cells and multicellular organisms", Chem. Biol. 15:427-437 (2008).
De Bruin et al., UBS Investment Research. Q-Series®: DNa Sequencing. UBS Securities LLC. Jul. 12, 2007. 15 pages.
Demirci, et al. Single cell epitaxy by acoustic picolitre droplets. Lab Chip. Sep. 2007;7(9):1139-45. Epub Jul. 10, 2007.
Doerr, "The smallest bioreactor", Nature Methods, 2:5 326 (2005).
Dowding, et al. Oil core/polymer shell microcapsules by interNal phase separation from emulsion droplets. II: controlling the release profile of active molecules. Langmuir. Jun. 7, 2005;21(12):5278-84.
Drmanac et al., Sequencing by hybridization (SBH): advantages, achievements, and opportunities. Adv Biochem Eng Biotechnol. 2002;77 :75-101.
Droplet Based Sequencing (slides) dated (Mar. 12, 2008).
Esser-Kahn, et al. Triggered release from polymer capsules. Macromolecules. 2011; 44:5539-5553.
European office action dated Jan. 23, 2012 for Application No. EP 08865992.5.

(56) **References Cited**

OTHER PUBLICATIONS

European office action dated Apr. 5, 2013 for Application No. EP 08865992.5.
European office action dated Aug. 29, 2013 for Application No. EP 08865992.5.
European office action dated Dec. 15, 2010 for EP Application No. 08865992.5.
Fisher, et al. A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. Genome Biol. 2011;12(1):R1. doi: 10.1186/gb-2011-12-1-r1. Epub Jan. 4, 2011.
Freiberg, et al. Polymer microspheres for controlled drug release. Int J Pharm. Sep. 10, 2004;282(1-2):1-18.
Fu, "A micro fabricated fluorescence-activated cell sorter", Nature Biotech., 17:1109-1111 (1997).
Fulton et al., Advanced multiplexed analysis with the FlowMetrix system. Clin Chem. Sep. 1997;43(9): 1749-56.
Garstecki, et al. Formation of monodisperse bubbles in a microfluidic flow-focusing device. Applied Physics Letters. 2004; 85(13):2649-2651. DOI: 10.1063/1.1796526.
Gartner, et al. The Microfluidic Toolbox—examples for fluidic interfaces and standardization concepts. Proc. SPIE 4982, Microfluidics, BioMEMS, and Medical Microsystems, (Jan. 17, 2003); doi: 10.1117/12.479566.
Ghadessy, et al. Directed evolution of polymerase function by compartmentalized self-replication. Proc Natl Acad Sci U S A. Apr. 10, 2001;98(8):4552-7. Epub Mar. 27, 2001.
Hashimshony, et al. CEL-Seq: Single-Cell RNa-Seq by Multiplexed Linear Amplification. Cell Rep. Sep. 27, 2012;2(3):666-73. doi: 10.1016/j.celrep.2012.08.003. Epub Aug. 30, 2012.
HE "Selective Encapsulation of Single Cells and Subcellular Organelles into Picoliter- and Femtoliter-Volume Droplets" ANal. Chem 77: 1539-1544 (2005).
Holtze, et al. Biocompatible surfactants for water-in-fluorocarbon emulsions. Lab Chip. Oct. 2008;8(10):1632-9. doi: 10.1039/b806706f. Epub Sep. 2, 2008.
Huebner, "Quantitative detection of protein expression in single cells using droplet microfluidics", Chern. Commun. 1218-1220 (2007).
Hug, et al. Measurement of the number of molecules of a single mRNA species in a complex mRNA preparation. J Theor Biol. Apr. 21, 2003;221(4):615-24.
International Preliminary Report on Patentability dated Mar. 31, 2011 for PCT/US09/005184.
International Preliminary Report on Patentability dated May 10, 2012 for PCT/US2010/054050.
International Preliminary Report on Patentability dated Jun. 30, 2011 for PCT/US2009/006649.
International Preliminary Report on Patentability dated Jul. 1, 2010 for PCT/US2008/013912.
International Preliminary Report on Patentability dated Sep. 17, 2009 for PCT/US2008/003185 mailed Sep. 17, 2009.
International search report and written opinion dated Jan. 12, 2009 for PCT/US2008/003185.
International search report and written opinion dated Jan. 31, 2011 for PCT/US2010/054050.
International search report and written opinion dated Apr. 3, 2009 for PCT/US2008/013912.
International search report and written opinion dated May 14, 2014 for PCT/US2014/015427.
International search report and written opinion dated May 16, 2014 for PCT/US2013/074764.
International search report and written opinion dated Aug. 16, 2010 for PCT/US2009/005184.
International search report and written opinion dated Aug. 20, 2014 for PCT/US2014/015424.
International search report and written opinion dated Oct. 2, 2009 for PCT/US2009/004037.
International search report and written opinion dated Oct. 21, 2009 for PCT/US2009/003389.
International search report and written opinion dated Oct. 29, 2008 for PCT/US2008/008563.
International search report and written opinion dated Dec. 16, 2013 for PCT/US2013/054797.
International search report dated Apr. 22, 2009 for PCT/US2009/000664.
Japanese Final Rejection dated Aug. 5, 2014 for Application No. JP 2012-536941.
Japanese Office Action and mailed Jul. 17, 2013 for Application No. JP 2010-539498.
Japanese Office Action mailed Nov. 19, 2013 for Application No. JP 2012-536941.
Khomiakov A et al., [Analysis of perfect and mismatched DNA duplexes by a generic hexanucleotide microchip]. Mol Bioi (Mosk). Jul.-Aug. 2003;37(4):726-41. Russian. Abstract only.
Kim, et al. Albumin loaded microsphere of amphiphilic poly(ethylene glycol)/poly(alpha-ester) multiblock copolymer. Eur J Pharm Sci. Nov. 2004;23(3):245-51.
Kim, et al. Fabrication of monodisperse gel shells and functioNal microgels in microfluidic devices. Angew Chem Int Ed Engl. 2007;46(11):1819-22.
Koster et al., "Drop-based microfluidic devices for encapsulation of single cells", Lab on a Chip The Royal Soc. of Chern. 8: 1110-1115 (2008).
Kutyavin, et al. Oligonucleotides containing 2-aminoadenine and 2-thiothymine act as selectively binding complementary agents. Biochemistry. Aug. 27, 1996;35(34):11170-6.
Li, Y., et al., "PEGylated PLGA Nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats," JourNal of Controlled Release, vol. 71, pp. 203-211 (2001).
Liu, et al. Preparation of uniform-sized PLA microcapsules by combining Shirasu porous glass membrane emulsification technique and multiple emulsion-solvent evaporation method. J Control Release. Mar. 2, 2005;103(1):31-43. Epub Dec. 21, 2004.
Liu, et al. Smart thermo-triggered squirting capsules for Nanoparticle delivery. Soft Matter. 2010; 6(16):3759-3763.
Loscertales, LG., et al., "Micro/Nano Encapsulation via Electrified Coaxial Liquid Jets," Science, vol. 295, pp. 1695-1698 (2002).
Love, "A microengraving method for rapid selection of single cells producing antigen-specific antibodies", Nature Biotech, 24:6 703 (Jun. 2006).
Marcus. Gene method offers diagnostic hope. The Wall Street JourNal. Jul. 11, 2012.
Mazutis, et al. Selective droplet coalescence using microfluidic systems. Lab Chip. Apr. 24, 2012;12(10):1800-6. doi: 10.1039/c21c40121e. Epub Mar. 27, 2012.
Merriman, et al. Progress in ion torrent semiconductor chip based sequencing. Electrophoresis. Dec. 2012;33(23):3397-417. doi: 10.1002/elps.201200424.
Microfluidic ChipShop. Microfluidic product catalogue. Mar. 2005.
Microfluidic ChipShop. Microfluidic product catalogue. Oct. 2009.
Mirzabekov, "DNA Sequencing by Hybridization—a Megasequencing Method and a Diagnostic Tool?" Trends in Biotechnology 12(1): 27-32 (1994).
Mouritzen et al., Single nucleotide polymorphism genotyping using locked nucleic acid (LNa). Expert Rev Mol Diagn. Jan. 2003;3(1):27-38.
Nguyen, et al. In situ hybridization to chromosomes stabilized in gel microdrops. Cytometry. 1995; 21:111-119.
Notice of Allowance dated Jan. 27, 2014 for U.S. Appl. No. 13/139,326.
Oberholzer, et al. Polymerase chain reaction in liposomes. Chem Biol. Oct. 1995;2(10):677-82.
Office action dated Jan. 4, 2010 for U.S. Appl. No. 12/172,186.
Office action dated Feb. 10, 2014 for U.S. Appl. No. 13/503,588.
Office action dated Feb. 28, 2013 for U.S. Appl. No. 13/139,326.
Office action dated Apr. 24, 2013 for U.S. Appl. No. 13/119,470.
Office action dated May 20, 2014 for U.S. Appl. No. 14/172,266.
Office action dated May 20, 2014 for U.S. Appl. No. 14/172,326.
Office action dated May 28, 2013 for U.S. Appl. No. 12/529,926.
Office action dated Jul. 30, 2014 for U.S. Appl. No. 12/809,120.
Office action dated Aug. 6, 2013 for U.S. Appl. No. 13/139,326.
Office action dated Aug. 6, 2014 for U.S. Appl. No. 12/529,926.

(56) **References Cited**

OTHER PUBLICATIONS

Office action dated Sep. 10, 2014 for U.S. Appl. No. 14/250,701.
Office action dated Sep. 17, 2013 for U.S. Appl. No. 13/503,588.
Office action dated Oct. 1, 2012 for U.S. Appl. No. 12/529,926.
Office action dated Dec. 5, 2013 for U.S. Appl. No. 13/119,470.
Office Action mailed Apr. 29, 2014 for EP Application No. 08865992.5.
Office Action mailed Dec. 16, 2013 for CN Application No. 201080055990.9.
Office Action mailed May 23, 2013 for CN Application No. 200880127116.4.
Ogawa, et al. Production and characterization of O/W emulsions containing cationic droplets stabilized by lecithin-chitosan membranes. J Agric Food Chem. Apr. 23, 2003;51(9):2806-12.
Okushima, "Controlled production of monodisperse double emulsions by two-step droplet breakup in microfluidic devices", Langmuir, 20:9905-9908 (2004).
Perez, C., et al., "Poly(lactic acid)-poly(ethylene glycol) Nanoparticles as new carriers for the delivery ofplasmid DNa," JourNal of Controlled Release, vol. 75, pp. 211-224 (2001).
Peters, et al. Accurate whole-genome sequencing and haplotyping from 10 to 20 human cells. Nature. Jul. 11, 2012;487(7406):190-5. doi: 10.1038/Nature11236.
Plunkett, et al. Chymotrypsin responsive hydrogel: application of a disulfide exchange protocol for the preparation of methacrylamide containing peptides. Biomacromolecules. Mar.-Apr. 2005;6(2):632-7.
Ryan, et al. Rapid assay for mycobacterial growth and antibiotic susceptibility using gel microdrop encapsulation. J Clin Microbiol. Jul. 1995;33(7):1720-6.
Schirinzi et al., Combinatorial sequencing-by-hybridization: aNalysis of the NFI gene. Genet Test. 2006 Spring;10(1):8-17.
Schmitt, "Bead-based multiplex genotyping of human papillomaviruses", J. Clinical Microbial., 44:2 504-512 (2006).
Shah, "Fabrication of mono disperse thermosensitive microgels and gel capsules in micro fluidic devices", Soft Matter, 4:2303-2309 (2008).
Simeonov et al., Single nucleotide polymorphism genotyping using short, fluorescently labeled locked nucleic acid (LNa) probes and fluorescence polarization detection. Nucleic Acids Res. Sep. 1, 2002;30(17):e91.
Sorokin et al., DiscrimiNation between perfect and mismatched duplexes with oligonucleotide gel microchips: role of thermodyNamic and kinetic effects during hybridization. J Biomol Struct Dyn. Jun. 2005;22(6):725-34.
Su, et al., Microfluidics-Based Biochips: Technology Issues, Implementation Platforms, and Design-Automation Challenges. IEEE Transactions on Computer-Aided Design of Integrated Circuits and Systems. 2006;25(2):211-23. (Feb. 2006).
Sun et al., Progress in research and application of liquid-phase chip technology. Chinese JourNal Experimental Surgery. May 2005;22(5):639-40.
Tawfik, et al. Man-made cell-like compartments for molecular evolution. Nat Biotechnol. Jul. 1998;16(7):652-6.
Theberge, et al. Microdropelts in microfluidics: an evolving platform for discoveries in chemsitry and biology. Angew Chem Int Ed Engl. Aug. 9, 2010;49(34):5846-68. doi: 10.1002/anie.200906653.
Tubeleviciute, et al. Compartmentalized self-replication (CSR) selection of Thermococcus litoralis Sh1B DNa polymerase for diminished uracil binding. Protein Eng Des Sel. Aug. 2010;23(8):589-97. doi: 10.1093/protein/gzq032. Epub May 31, 2010.
Wang et al., Single nucleotide polymorphism discrimiNation assisted by improved base stacking hybridization using oligonucleotide microarrays. Biotechniques. 2003;35:300-08.
Wang, et al. A novel thermo-induced self-bursting microcapsule with magnetic-targeting property. Chemphyschem. Oct. 5, 2009;10(14):2405-9.
Weaver, "Rapid clonal growth measurements at the single-cell level: gel microdroplets and flow cytometry", Biotechnology, 9:873-877 (1991).
Whitesides, "Soft lithography in biology and biochemistry", Annual Review of Biomedical Engineering, 3:335-373 (2001).
Williams, et al. Amplification of complex gene libraries by emulsion PCR. Nat Methods. Jul. 2006;3(7):545-50.
Woo, et al. G/C-modified oligodeoxynucleotides with selective complementarity: synthesis and hybridization properties. Nucleic Acids Res. Jul. 1, 1996;24(13):2470-5.
Xia, "Soft lithography", Annual Review of Material Science, 28: 153-184 (1998).
Yamamoto, et al. Chemical modification of Ce(IV)/EDTA-base artificial restriction DNa cutter for versatile manipulation of doulbestranded DNa. Nucleic Acids Research. 2007; 35(7):e53.
Zhang, "Combinatorial marking of cells and organelles with reconstituted fluorescent proteins", Cell, 119:137-144 (Oct. 1, 2004).
Zhang, et al. Degradable disulfide core-cross-linked micelles as a drug delivery system prepared from vinyl functioNalized nucleosides via the RAFT process. Biomacromolecules. Nov. 2008;9(11):3321-31. doi: 10.1021/bm800867n. Epub Oct. 9, 2008.
Zhao, J., et al., "Preparation of hemoglobin-loaded Nano-sized particles with porous structure as oxygen carriers," Biomaterials, vol. 28, pp. 1414-1422 (2007).
Zimmermann et at., Microscale production of hybridomas by hypo-osmolar electrofusion. Hum. Antibodies Hybridomas. Jan. 1992;3(1): 14-8.
Zong, et al. Genome-wide detection of single-nucleotide and copy-number variations of a single human cell. Science. Dec. 21, 2012;338(6114):1622-6. doi: 10.1126/science.129164.
Office action dated Jan. 15, 2015 for U.S. Appl. No. 14/250,701.
Gyarmati, et al. Reversible disulphide formation in polymer networks: a versatile functional group from synthesis to applications. European Polymer Journal. 2013; 49:1268-1286.
Office action dated Apr. 20, 2015 for U.S. Appl. No. 13/966,150.
Office action dated Feb. 23, 2016 for U.S. Appl. No. 14/104,650.
Office action dated Mar. 1, 2016 for U.S. Appl. No. 14/250,701.
Office action dated Mar. 4, 2016 for U.S. Appl. No. 14/175,973.
Office action dated Mar. 14, 2016 for U.S. Appl. No. 14/680,808.
Shimkus, et al. A chemically cleavable biotinylated nucleotide: usefulness in the recovery of protein-DNA complexes from avidin affinity columns Proc Natl Acad Sci U S A. May 1985;82(9):2593-7.
European search report and opinion dated Feb. 2, 2016 for EP Application No. 13829413.
Office action dated Jan. 29, 2016 for U.S. Appl. No. 14/175,935.
Aitman, et al. Copy number polymorphism in Fcgr3 predisposes to glomerulonephritis in rats and humans. Nature. Feb. 16, 2006;439(7078):851-5.
Balikova, et al. Autosomal-dominant microtia linked to five tandem copies of a copy-number-variable region at chromosome 4p16. Am J Hum Genet. Jan. 2008;82(1):181-7. doi: 10.1016/j.ajhg.2007.08.001.
Browning, et al. Haplotype phasing: existing methods and new developments. Nat Rev Genet. Sep. 16, 2011;12(10):703-14. doi: 10.1038/nrg3054. Review.
Cappuzzo, et al. Increased HER2 gene copy number is associated with response to gefitinib therapy in epidermal growth factor receptor-positive non-small-cell lung cancer patients. J Clin Oncol. Aug. 1, 2005;23(22):5007-18.
Choi, et al. Identification of novel isoforms of the EML4-ALK transfrorming gene in non-small cell lung cancer. Cancer Res. Jul. 1, 2008;68(13):4971-6. doi: 10.1158/0008-5472.CAN-07-6158.
Cook, et al. Copy-number variations associated with neuropsychiatric conditions. Nature. Oct. 16, 2008;455(7215):919-23. doi: 10.1038/nature07458.
Co-pending U.S. Appl. No. 15/392,557, filed Dec. 28, 2016.
"Dressman et al. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. Proc. Natl. Acad. Sci. 2003. 100(15):8817-882."
Fabi, et al. Correlation of efficacy between EGFR gene copy number and lapatinib/capecitabine therapy in HER2-positive metastatic breast cancer. J. Clin. Oncol. 2010; 28:15S. 2010 ASCO Meeting abstract Jun. 14, 2010:1059.

(56) **References Cited**

OTHER PUBLICATIONS

Gonzalez, et al. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science. Mar. 4, 2005;307(5714):1434-40. Epub Jan. 6, 2005.

"Sigma. Streptavidin-agarose (S1638) product information sheet. www.sigma-aldrich.com".

Kitzman, et al. Noninvasive whole-genome sequencing of a human fetus. Sci Transl Med. Jun. 6, 2012;4(137):137ra76. doi: 10.1126/scitranslmed.3004323.

Knight, et al. Subtle chromosomal rearrangements in children with unexplained mental retardation. Lancet. Nov. 13, 1999(9191):1676-81.

Lupski. Genomic rearrangements and sporadic disease. Nat Genet. Jul. 2007;39(7 Suppl):S43-7.

Pinto, et al. Functional impact of global rare copy number variation in autism spectrum disorders. Nature. Jul. 15, 2010(7304);368-72. doi: 10.1038/nature09146. Epub Jun. 9, 2010.

Ropers. New perspectives for the elucidation of genetic disorders. Am J Hum Genet. Aug. 2007;81(2):199-207. Epub Jun. 29, 2007.

Sebat, et al. Strong association of de novo copy number mutations with autism. Science. Apr. 20, 2007;316(5823):445-9. Epub Mar. 15, 2007.

Shlien, et al. Copy number variations and cancer. Genome Med. Jun. 16, 2009;1(6):62. doi: 10.1186/gm62.

Shlien, et al. Excessive genomic DNA copy number variatioin in the Li-Fraumeni cancer predisposition syndrome. Proc Natl Acad Sci U S A. Aug. 12, 2008(32):11264-9. doi: 10.1073/pnas.0802970105. Epub Aug. 6, 2008.

Wang, et al. Digital Karyotyping. Proc Natl Acad Sci U S A. Dec. 10, 2002;99(25):16156-61. Epub Dec. 2, 2002.

Co-pending U.S. Appl. No. 15/440,772, filed Feb. 23, 2017.

Co-pending U.S. Appl. No. 15/449,741, filed Mar. 3, 2017.

Abate, et al. Beating Poisson encapsulation statistics using close-packed ordering. Lab Chip. Sep. 21, 2009;9(18):2628-31. doi: 10.1039/b909386a. Epub Jul. 28, 2009.

Altemos et al., "Genomic Characterization of Large Heterochromatic Gaps in the Human Genome Assembly," PLOS Computational Biology, May 15, 2014, vol. 10, Issue 5, 14 pages.

Amini, S. et al. "Haplotype-resolved whole-genome sequencing by contiguity-preserving transposition and combinatorial indexing" Nature Genetics (2014) 46:1343-1349 doi:10.1038/ng.3119.

Anonymous, "Oligo(dT)25 cellulose beads" NEB (2012) Retrieved from the Internet:https://www.neb.com/~/media/Catalog/All-Products/286CA51268E24DE1B06F1CB288698B54/Datacards%20or%Manuals/S1408Datasheet-Lot0011205.pdf.

Anonymous, "Oligotex Handbook" Qiagen (2012) XP055314680, Retrieved from the Internet: URL:http://www.qiagen.com/de/resources/download.apsx?id=f9fald98-d54d-47e7-a20b-8b0cb8975009&lang=en.

Anonymous: "Viscosity-Basic concepts" (2004) XP055314117, Retrieved from the Internet: URL:http://lhtc.epfl.ch/webdav/site/lhtc/shared/import/migration/2 VISCOSITY.pdf.

Attia, et al. Micro-injection moulding of polymer microfluidic devices. Microfluidics and nanofluidics. 2009; 7(1):1-28.

Baret, et al. Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. Lab Chip. Jul. 7, 2009;9(13):1850-8. doi: 10.1039/b902504a. Epub Apr. 23, 2009.

Bodi, K. et al. "Comparison of Commercially Available Target Enrichment Methods for Next-Generation Sequencing" J Biomolecular Techniques (2013) 24:73-86.

Brouzes, et al. Droplet microfluidic technology for single-cell high-throughput screening. Proc Natl Acad Sci U S A. Aug. 25, 2009;106(34):14195-200. doi: 10.1073/pnas.0903542106. Epub Jul. 15, 2009.

Chen, et al. Chemical transfection of cells in picoliter aqueous droplets in fluorocarbon oil. Anal Chem. Nov. 15, 2011;83(22):8816-20. doi: 10.1021/ac2022794. Epub Oct. 17, 2011.

Chokkalingam, et al. Probing cellular heterogeneity in cytokine-secreting immune cells using droplet-based microfluidics. Lab Chip. Dec. 21, 2013;13(24):4740-4. doi: 10.1039/c3lc50945a.

Christiansen et al. "The Covalent Eukaryotic Topoisomerase I-DNA Intermediate Catalyzes pH-dependent Hydrolysis and Alcoholysis" J Biol Chem (Apr. 14, 1994) 269(15):11367-11373.

Chu, et al. Controllable monodisperse multiple emulsions. Angew Chem Int Ed Engl. 2007;46(47):8970-4.

Coufal, et al. L1 retrotransposition in human neural progenitor cells. Nature. Aug. 27, 2009;460(7259):1127-31. doi: 10.1038/nature08248. Epub Aug. 5, 2009.

Doshi, et al. Red Blood cell-mimicking synthetic biomaterial particles. Proceedings of the National Academy of Sciences 106.51 (2009): 21495-21499.

Draper, et al. Compartmentalization of electrophoretically separated analytes in a multiphase microfluidic platform. Anal Chem. Jul. 3, 2012;84(13):5801-8. doi: 10.1021/ac301141x. Epub Jun. 13, 2012.

Dressler, et al. Droplet-based microfluidics enabling impact on drug discovery. J Biomol Screen. Apr. 2014;19(4):483-96. doi: 10.1177/1087057113510401. Epub Nov. 15, 2013.

"Eastburn, et al. Ultrahigh-throughput mammalian single-cell reverse-transcriptase polymerase chain reaction in microfluidic droplets. Anal Chem. Aug. 20, 2013;85(16):8016-21. doi: 10.1021/ac402057q. Epub Aug. 8, 2013."

Fan, et al. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci U S A. Oct. 21, 2008;105(42):16266-71. doi: 10.1073/pnas.0808319105. Epub Oct. 6, 2008.

Fang, et al. Fluoride-cleavable biotinylation phosphoramidite for 5'-end-labeling and affinity purification of synthetic oligonucleotides. Nucleic Acids Res. Jan. 15, 2003;31(2):708-15.

Frampton, G.M. et al. "Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing" Nature Biotechnology (2013) 31(11):1023-1031. doi:10.1038/nbr.2696.

Fredreickson, et al. Macro-to-micro interfaces for microfluidic devices. Lab Chip. Dec. 2004;4(6):526-33. Epub Nov. 10, 2004.

Fu, A.Y., et al., "A microfabricated fluorescence-activated cell sorter," Nature Biotechnology, vol. 17, pp. 1109-1111 (1999).

Gericke, et al. Functional cellulose beads: preparation, characterization, and applications. Chemical reviews 113.7 (2013): 4812-4836.

Granieri, Lucia. Droplet-based microfluidics and engineering of tissue plasminogen activator for biomedical applications. Ph.D. Thesis, Nov. 13, 2009 (131 pages).

Grasland-Mongrain, et al. Droplet coalescence in microfluidic devices. Jan.-Jul. 2003. 31 pages. http://www.eleves.ens.fr/home/grasland/rapports/stage4.pdf.

Guo, et al. Droplet microfluidics for high-throughput biological assays. Lab Chip. Jun. 21, 2012;12(12):2146-55. doi: 10.1039/c2lc21147e. Epub Feb. 9, 2012.

He, J. et al. "Genotyping-by-sequencing (GBS), an ultimate marker-assisted selections (MAS) tool to accelerate plant breeding" Frontiers in Plant Sci (Sep. 30, 2014) 5:1-8.

Hjerten, et al. General methods to render macroporous stationary phases nonporous and deformable, exemplified with agarose and silica beads and their use in high-performance ion-exchange and hydrophobic-interaction chromatography of proteins. Chromatographia 31.1-2 (1991): 85-94.

Ioannidis, N. Manufacturing of Agarose-Based Chromatographic Adsorbents with Controlled Pore and Particel Sizes. A thesis submitted to The University of Birmingham for the degree of Doctor of Philosophy. 2009.

Jena, et al. Cyclic olefin copolymer based microfluidic devices for biochip applications: Ultraviolet surface grafting using 2-methacryloyloxyethyl phosphorylcholine. Biomicrofluidics. Mar. 2012;6(1):12822-1282212. doi: 10.1063/1.3682098. Epub Mar. 15, 2012.

Jung, et al. Micro machining of injection mold inserts for fluidic channel of polymeric biochips. Sensors. 2007; 7(8):1643-1654.

Kim, et al. Rapid prototyping of microfluidic systems using a PDMS/polymer tape composite. Lab Chip. May 7, 2009;9(9):1290-3. doi: 10.1039/b818389a. Epub Feb. 10, 2009.

Kleiin, et al. Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. Cell. May 21, 2015; 161(5):1187-201. doi: 10.1016/j.cell.2015.04.044.

(56) **References Cited**

OTHER PUBLICATIONS

Lagus, et al. A review of the theory, methods and recent applications of high-throughput single-cell droplet microfluidics. J. Phys. D: Appl. Phys. (2013) 46:114005. (21 pages).
Lee, J-H. et al. "Fluorescent in situ sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues" Nature Protocols (Feb. 12, 2015) 10(3):442-458.
Lowe, Adam J. Norbornenes and [n]polynorbornanes as molecular scaffolds for anion recognition. Ph.D. Thesis (May 2010). (361 pages).
Macosko, et al. Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. Cell. May 21, 2015;161(5):1202-14. doi: 10.1016/j.cell.2015.05.002.
Mair, et al. Injection molded microfluidic chips featuring integrated interconnects. Lab Chip. Oct. 2006;6(10):1346-54. Epub Jul. 31, 2006.
Makino, et al. Preparation of hydrogel microcapsules: Effects of preparation conditions upon membrane properties. Colloids and Surfaces B: Biointerfaces. Nov. 1998; 12(2), 97-104.
Matochko, et al. Uniform amplification of phage display libraries in monodisperse emulsions. Methods. Sep. 2012;58(1):18-27. doi: 10.1016/j.ymeth.2012.07.012. Epub Jul. 20, 2012.
Moore, et al. Behavior of capillary valves in centrifugal microfluidic devices prepared by three-dimensional printing. Microfluidics and Nanofluidics. 2011; 10(4):877-888.
Morgan, et al. Chapter 12: Human microbiome analysis. PLoS Comput Biol. 2012;8(12):e1002808. doi: 10.1371/journal.pcbi.1002808. Epub Dec. 27, 2012.
Mozhanova, A.A. et al. "Local elastic properties of biological materials studied by SFM" (2003) XP055314108, Retrieved from the Internet: URL:http://www.ntmdt.com/data/media/files/publications/2003/08.08_a.a.mozhanova_n.i.n_enlish.pdf.
Muotri, et al. L1 retrotransposition in neurons is modulated by MeCP2. Nature. Nov. 18, 2010;468(7322):443-6. doi: 10.1038/nature09544.
Nagashima, et al. Preparation of monodisperse poly (acrylamide-co-acrylic acid) hydrogel microspheres by a membrane emulsification technique and their size-dependent surface properties. Colloids and Surfaces B: Biointerfaces. Jun. 15, 1998; 11(1-2), 47-56.
Navin. The first five years of single-cell cancer genomics and beyond. Genome Res. Oct. 2015;25(10):1499-507. doi: 10.1101/gr.191098.115.
Novak, et al. Single cell multiplex gene detection and sequencing using microfluidicallygenerated agarose emulsions. Angew Chem Int Ed Engl. Jan. 10, 2011;50(2):390-5. doi: 10.1002/anie.201006089.
Oligotex Handbook. For purification of poly A+ RNA from total RNA and directly from cultured cells or tissues as well as purification of polyadenylated in vitro transcripts. Jun. 2012.
Pantel, et al. Detection methods of circulating tumor cells. J Thorac Dis. Oct. 2012;4(5):446-7. doi: 10.3978/j.issn.2072-1439.2012.08.15.
Patel, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. Science. Jun. 20, 2014;344(6190):1396-401. doi: 10.1126/science.1254257. Epub Jun. 12, 2014.
Picot, J. et al. "A biomimetic microfluidic chip to study the circulation and mechanical retention of red blood cells in the spleen" Am J Hematology (Jan. 12, 2015) 90(4):339-345.
Ram, et al. Strategy for microbiome analysis using 16S rRNA gene sequence analysis on the Illumiina sequencing platform. Syst Biol Reprod Med. Jun. 2011;57(3):162-70. doi: 10.3109/19396368.2011.555598. Epub Mar. 1, 2011.
Richardson, et al. Novel inhibition of archaeal family-D DNA polymerase by uracil. Nucleic acids research 41.7 (2013): 4207-4218.
Rogozin, et al. A highly conserved family of inactivated archaeal B family DNA polymerases. Biol Direct. Aug. 6, 2008;3:32. doi: 10.1186/1745-6150-3-32.
Rotem, et al. High-Throughput Single-Cell Labeling (Hi-SCL) for RNA-Seq Using Drop-Based Microfluidics. PLoS One. May 22, 2015;10(5):e0116328. doi: 10.1371/journal.pone.0116328. eCollection 2015.
Rotem, et al. Single Cell Chip-Seq Using Drop-Based Microfluidics. Abstract #50. Frontiers of Single Cell Analysis, Stanford University Sep. 5-7, 2013.
Schmeider, et al. Fast identification and removal of sequence contamination from genomic and metagenomic datasets. PLoS One. Mar. 9, 2011;6(3):e17288. doi: 10.1371/journal.pone.0017288.
Seiffert, et al. Smart microgel capsules from macromolecular precursors. J Am Chem Soc. May 12, 2010;132(18):6606-9. doi:10.1021/ja102156h.
Shuttleworth, et al. Recognition of the pro-mutagenic base uracil by family B DNA polymerases from archaea. J Mol Biol. Mar. 26, 2004;337(3):621-34.
Tayyab, S. et al. "Size exclusion chromatography and size exclusion HPLC of proteins" Biochem Ed, Pergamon, (1991) 1993):149-152.
Tewhey, et al. Microdroplet-based PCR enrichment for large-scale targeted sequencing. Nat Biotechnol. Nov. 2009;27(11):1025-31. doi: 10.1038/nbt.1583. Epub Nov. 1, 2009.
Tonelli, et al. Perfluoropolyether functional oligomers: unusual reactivity in organic chemisty. Journal of fluorine chemistry. 2002; 118(1)"107-121.
Turner, et al. Methods for genomic partitioning. Annu Rev Genomics Hum Genet. 2009;10:263-84. doi: 10.1146/annurev-genom-082908-150112. Review.
Wagner, et al. Biocompatible fluorinated polyglycerols for droplet microfluidics as an alternative to PEG-based copolymer surfactants. Lab Chip. Jan. 7, 2016;16(1):65-9. doi: 10.1039/c5lc00823a. Epub Dec. 2, 2015.
Zheng, X.Y. et al. "Haplotyping germline and cancer genomes with high-throughput linked-read sequencing" Nature Biotech (Feb. 1, 2016) 34(3):303-311.
Zhu, et al. Synthesis and self-assembly of highly incompatible polybutadienepoly(hexafluoropropoylene oxide) diblock copolymers. Journal Polymer Science Part B: Polymer Physics. 2005; 43(24):3685-3694.
Berkum, et al. Hi-C: a method to study the three-dimensional architecture of genomes. J Vis Exp. May 6, 2010;(39). pii: 1869. doi: 10.3791/1869.
Chung, et al. Structural and molecular interrogation of intact biological systems. Nature. May 16, 2013; 497(7449):332-7. doi: 10.1038/nature12107. Epub Apr. 10, 2013.
Dekker, et al. Capturing chromosome conformation. Science. Feb. 15, 202;295(5558):1306-11.
Illumina Nextera Enrichment Sample Preparation Guide. Feb. 2013.
Illumina TruSeq Custom Enrichment Kit Data Sheet. (c) 2014.
Kaper, et al. Supporting Information for "Whole-genome haplotyping by dilution, amplification, and sequencing." Proc Natl Acad Sci U S A. Apr. 2, 2013;110(14):5552-7. doi: 10.1073/pnas.1218696110. Epub Mar. 18, 2013.
Kaper, et al. Whole-genome haplotyping by dilution, amplification, and sequencing. Proc Natl Acad Sci U S A. Apr. 2, 2013;110(14):5552-7. doi: 10.1073/pnas.1218696110. Epub Mar. 18, 2013.
Karmaker, et al. Organocatalytic removal of formaldehyde adducts from RNA and DNA bases. Nat Chem. Sep. 2015;7(9):752-8. doi: 10.1038/nchem.2307. Epub Aug. 3, 2015.
Kebschull, et al. High-Throughput Mapping of Single-Neuron Projections by Sequencing of Barcoded RNA. Neuron. Sep. 7, 2016;91(5):975-87. doi: 10.1016/j.neuron.2016.07.036. Epub Aug. 18, 2016.
Lee, et al. Act-Presto: Rapid and consistent tissue clearing and labeling method for 3-dimensional (3D) imaging. Sci Rep. Jan. 11, 2016;6:18631. doi: 10.1038/srep18631.
Susaki, et al. Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. Cell. Apr. 24, 2014;157(3):726-39. doi: 10.1016/j.cell.2014.03.042. Epub Apr. 17, 2014.
Tomer, et al. Advanced CLARITY for rapid and high-resolution imaging of intact tissues. Nat Protoc. Jul. 2014;9(7):1682-97. doi: 10.1038/nprot.2014.123. Epub Jun. 19, 2014.

(56) **References Cited**

OTHER PUBLICATIONS

Wesolowska, et al. Cost-effective multiplexing before capture allows screening of 25 000 clinically relevant SNPs in childhood acute lymphoblastic leukemia. Leukemia. Jun. 2011;25(6):1001-6. doi: 10.1038/leu.2011.32. Epub Mar. 18, 2011.
Co-pending U.S. Appl. No. 15/430,298, filed Feb. 10, 2017.
Co-pending U.S. Appl. No. 15/470,814, filed Mar. 27, 2017.
Co-pending U.S. Appl. No. 15/598,898, filed May 18, 2017.
Stoeckius, et al. Large-scale simultaneous measurement of epitopes and transcriptomes in single cells. bioRxiv 113068; doi: https://doi.org/10.1101/113068.

* cited by examiner

Fig. 1A

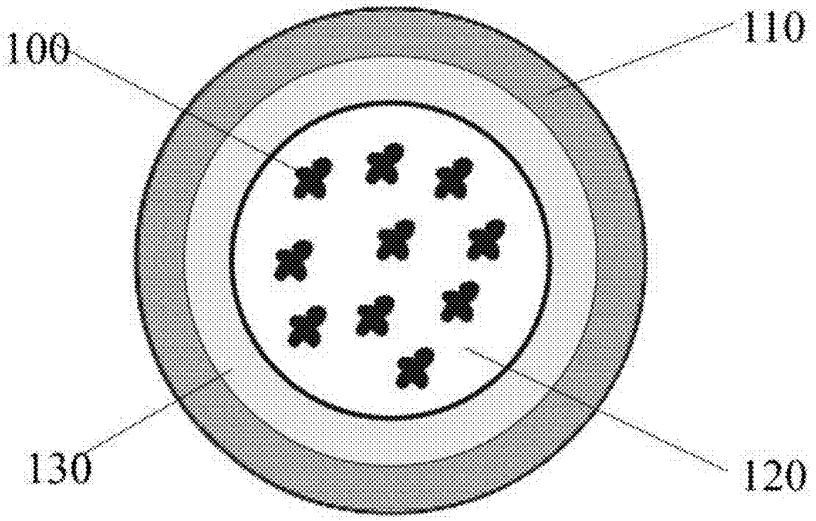


Fig. 1B

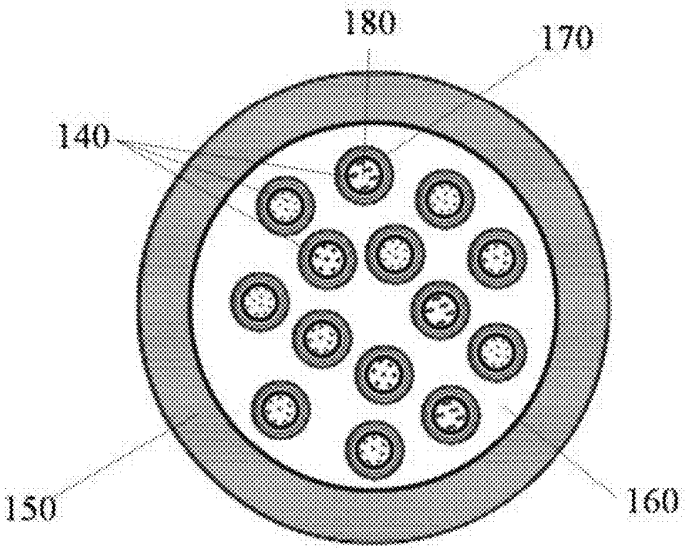


Fig. 2A

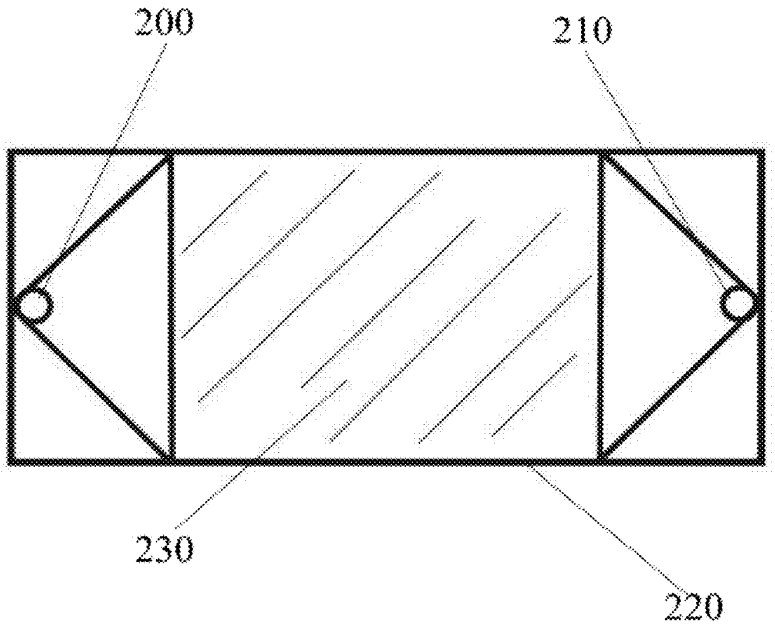


Fig. 2B

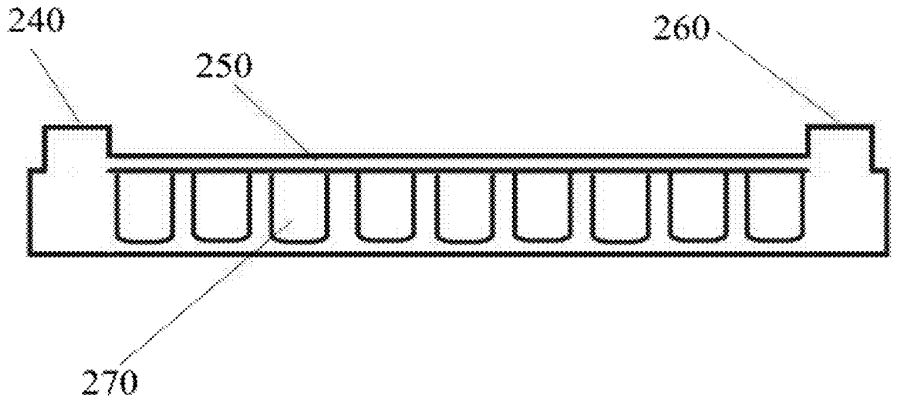


Fig. 3

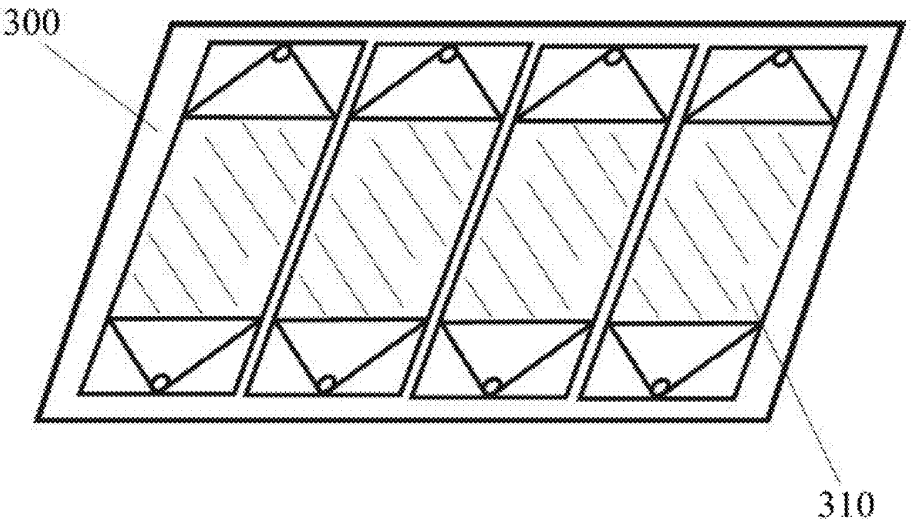


Fig. 4A

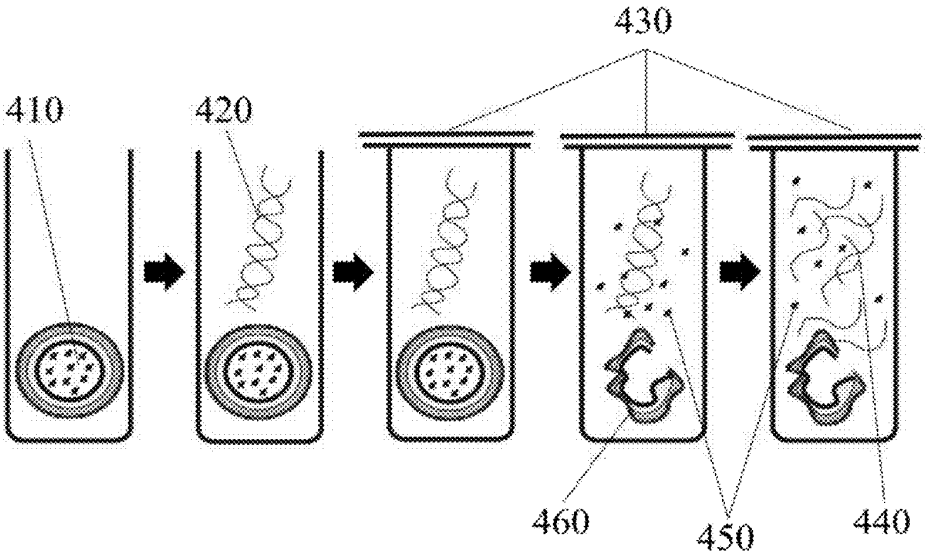
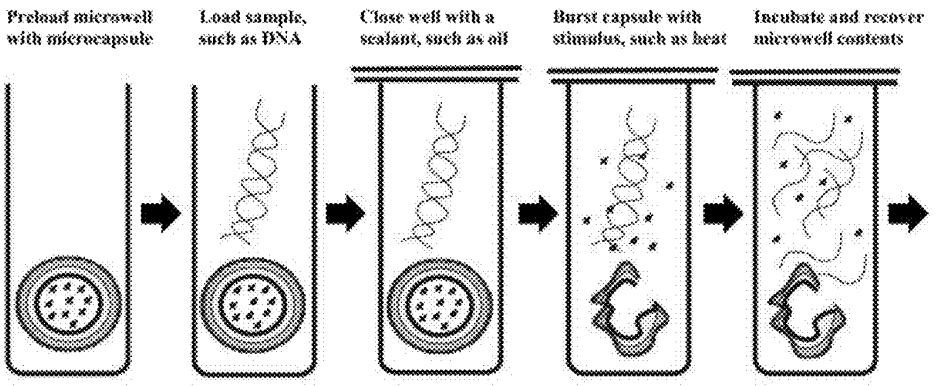

430
410
420
460
450
440

Fig. 4B


Preload microwell with microcapsule
Load sample, such as DNA
Close well with a sealant, such as oil
Burst capsule with stimulus, such as heat
Incubate and recover microwell contents

METHODS FOR DROPLET-BASED SAMPLE PREPARATION

CROSS-REFERENCE

This application is a divisional of U.S. patent application Ser. No. 13/966,150, filed Aug. 13, 2013, which applications claim the benefit of U.S. Provisional Patent Application No. 61/683,192, filed Aug. 14, 2012; U.S. Provisional Patent Application No. 61/737,374, filed Dec. 14, 2012; U.S. Provisional Patent Application No. 61/762,435, filed Feb. 8, 2013; U.S. Provisional Patent Application No. 61/800,223, filed Mar. 15, 2013; U.S. Provisional Patent Application No. 61/840,403, filed Jun. 27, 2013; and U.S. Provisional Patent Application No. 61/844,804, filed Jul. 10, 2013, which applications are incorporated herein by reference in their entireties for all purposes.

BACKGROUND OF THE INVENTION

The detection and quantification of analytes is important for molecular biology and medical applications such as diagnostics. Genetic testing is particularly useful for a number of diagnostic methods. For example, disorders that are caused by mutations, such as cancer, may be detected or more accurately characterized with DNA sequence information.

Appropriate sample preparation is often needed prior to conducting a molecular reaction such as a sequencing reaction. A starting sample may be a biological sample such as a collection of cells, tissue, or nucleic acids. When the starting material is cells or tissue, the sample may need to be lysed or otherwise manipulated in order to permit the extraction of molecules such as DNA. Sample preparation may also involve fragmenting molecules, isolating molecules, and/or attaching unique identifiers to particular fragments of molecules, among other actions. There is a need in the art for improved methods and devices for preparing samples prior to downstream applications.

SUMMARY OF THE INVENTION

This disclosure provides compositions and methods for a microcapsule array device.

An aspect of the disclosure provides a composition comprising a first microcapsule, wherein: the first microcapsule is degradable upon the application of a stimulus to the first microcapsule; and the first microcapsule comprises an oligonucleotide barcode. In some cases, the first microcapsule may comprise a chemical cross-linker. The chemical cross-linker, for example, may be a disulfide bond. In some cases, the composition may comprise a polymer gel, such as, for example a polyacrylamide gel. The first microcapsule may comprise a bead. In some cases, the bead may be a gel bead.

Moreover, the stimulus may be selected from the group consisting of a biological, chemical, thermal, electrical, magnetic, or photo stimulus, and combination thereof. In some cases, the chemical stimulus may be selected from the group consisting of a change in pH, a change in ion concentration, and a reducing agent. The reducing agent may be, for example, dithiothreitol (DTT) or tris(2-carboxyethyl) phosphine (TCEP).

A second microcapsule may comprise the first microcapsule. Moreover, the second microcapsule may be a droplet. In some cases, the composition may also comprise a nucleic acid that comprises the oligonucleotide barcode, wherein the nucleic acid comprises a deoxyuridine triphosphate (dUTP).

In some cases, the composition may comprise a polymerase unable to accept a deoxyuridine triphosphate (dUTP). Also, the composition may comprise a target analyte, such as, for example, a nucleic acid. The nucleic acid may be selected from the group consisting of DNA, RNA, dNTPs, ddNTPs, amplicons, synthetic nucleotides, synthetic polynucleotides, polynucleotides, oligonucleotides, peptide nucleic acids, cDNA, dsDNA, ssDNA, plasmid DNA, cosmid DNA, High Molecular Weight (MW) DNA, chromosomal DNA, genomic DNA, viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), mRNA, rRNA, tRNA, nRNA, siRNA, snRNA, snoRNA, scaRNA, microRNA, dsRNA, ribozyme, riboswitch and viral RNA. In some cases, the nucleic acid may be genomic DNA (gDNA).

Additionally, the density of the oligonucleotide barcodes may be at least about 1,000,000 oligonucleotide barcodes per the first microcapsule. The oligonucleotide barcode may be coupled to the microcapsule via a chemical cross-linker, such as, for example a disulfide bond.

An additional aspect of the disclosure comprises a device comprising a plurality of partitions, wherein: at least one partition of the plurality of partitions comprises a microcapsule comprising an oligonucleotide barcode; and the microcapsule is degradable upon the application of a stimulus to the microcapsule. The partition, for example, may be a well or a droplet. In some cases, the microcapsule comprises a chemical cross-linker such as, for example, a disulfide bond. Moreover, the microcapsule may comprise a polymer gel such as, for example, a polyacrylamide gel. Also, the microcapsule may comprise a bead. In some cases, the bead may be a gel bead.

The stimulus may be selected from the group consisting of a biological, chemical, thermal, electrical, magnetic, or photo stimulus, and a combination thereof. In some cases, the chemical stimulus may be selected from the group consisting of a change in pH, change in ion concentration, and a reducing agent. The reducing agent, for example, may be dithiothreitol (DTT) or tris(2-carboxyethyl) phosphine (TCEP).

Furthermore, a nucleic acid may comprise the oligonucleotide barcode and the nucleic acid may comprise a deoxyuridine triphosphate (dUTP). In some cases, the partition may comprise a polymerase unable to accept a deoxyuridine triphosphate (dUTP). Additionally, the partition may comprise a target analyte such as, for example, a nucleic acid. The nucleic acid may be selected from the group consisting of DNA, RNA, dNTPs, ddNTPs, amplicons, synthetic nucleotides, synthetic polynucleotides, polynucleotides, oligonucleotides, peptide nucleic acids, cDNA, dsDNA, ssDNA, plasmid DNA, cosmid DNA, High Molecular Weight (MW) DNA, chromosomal DNA, genomic DNA, viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), mRNA, rRNA, tRNA, nRNA, siRNA, snRNA, snoRNA, scaRNA, microRNA, dsRNA, ribozyme, riboswitch and viral RNA. In some cases, the nucleic acid may be genomic DNA (gDNA). The oligonucleotide barcode may be coupled to the microcapsule via a chemical cross-linker. In some cases, the chemical cross-linker may be a disulfide bond.

A further aspect of the disclosure provides a method for sample preparation comprising combining a microcapsule comprising an oligonucleotide barcode and a target analyte into a partition, wherein the microcapsule is degradable upon the application of a stimulus to the microcapsule; and applying the stimulus to the microcapsule to release the oligonucleotide barcode to the target analyte. The partition may be, for example, a well or a droplet. In some cases, the microcapsule may comprise a polymer gel such as, for

example, a polyacrylamide. Moreover, the microcapsule may comprise a bead. In some cases, the bead may be a gel bead. Moreover, the microcapsule may comprise a chemical cross-linker such as, for example, a disulfide bond.

The stimulus may be selected from the group consisting of a biological, chemical, thermal, electrical, magnetic, photo stimulus, and a combination thereof. In some cases, the chemical stimulus may be selected from the group consisting of a change in pH, change in ion concentration, and a reducing agent. The reducing agent may be, for example, dithiothreitol (DTT) or tris(2-carboxyethyl) phosphine (TCEP).

Also, a nucleic acid may comprise the oligonucleotide barcode and the nucleic acid may comprise a deoxyuridine triphosphate (dUTP). In some cases, the partition may comprise a polymerase unable to accept a deoxyuridine triphosphate (dUTP). Moreover, the method may also comprise attaching the oligonucleotide barcode to the target analyte. The attaching may be completed, for example, with a nucleic acid amplification reaction. Moreover, the analyte may be a nucleic acid. In some cases, the nucleic acid may be selected from the group consisting of DNA, RNA, dNTPs, ddNTPs, amplicons, synthetic nucleotides, synthetic polynucleotides, polynucleotides, oligonucleotides, peptide nucleic acids, cDNA, dsDNA, ssDNA, plasmid DNA, cosmid DNA, High Molecular Weight (MW) DNA, chromosomal DNA, genomic DNA, viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), mRNA, rRNA, tRNA, nRNA, siRNA, snRNA, snoRNA, scaRNA, microRNA, dsRNA, ribozyme, riboswitch and viral RNA. In some cases, the nucleic acid may be genomic DNA (gDNA). Furthermore, the oligonucleotide barcode may be coupled to the microcapsule via a chemical cross-linker. In some cases, the chemical cross-linker may be a disulfide bond.

A further aspect of the disclosure provides a composition comprising a degradable gel bead, wherein the gel bead comprises at least about 1,000,000 oligonucleotide barcodes. In some cases, the 1,000,000 oligonucleotide barcodes are identical.

INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference in their entireties for all purposes and to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

The novel features of a device of this disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of this disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of a device of this disclosure are utilized, and the accompanying drawings of which:

FIG. **1**A is a schematic representation of a microcapsule or inner reagent droplet.

FIG. **1**B is a schematic representation of a microcapsule containing multiple inner reagent droplets.

FIG. **2**A is a schematic illustration of a top down view of an exemplary microcapsule array.

FIG. **2**B is a schematic illustration of an exemplary side view of a microcapsule array.

FIG. **3** is a schematic illustration of a multi-microcapsule array configuration on a 96-well plate holder.

FIG. **4**A is a schematic flow diagram representative of a reaction sequence in one microwell of a microwell capsule array.

FIG. **4**B is similar to **4**A, except that it is annotated with examples of methods that can be performed at each step.

DETAILED DESCRIPTION OF THE INVENTION

While various embodiments of the invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions may occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed.

I. General Overview

The present disclosure provides microwell or other partition capsule array devices and methods of using such devices. Generally, the device is an assembly of partitions (e.g., microwells, droplets) that are loaded with microcapsules, often at a particular concentration of microcapsules per partition.

The devices may be particularly useful to perform sample preparation operations. In some cases, a device subdivides a sample (e.g., a heterogeneous mixture of nucleic acids, a mixture of cells, etc.) into multiple partitions such that only a portion of the sample is present in each partition. For example, a nucleic acid sample comprising a mixture of nucleic acids may be partitioned such that no more than one strand of (or molecule of) nucleic acid is present in each partition. In other examples, a cell sample may be partitioned such that no more than one cell is present in each partition.

Following the partitioning step, any of a number of different operations may be performed on the subdivided sample within the device. The partitions may include one or more capsules that contain one or more reagents (e.g., enzymes, unique identifiers (e.g., bar codes), antibodies, etc.). In some cases, the device, a companion device or a user provides a trigger that causes the microcapsules to release one or more of the reagents into the respective partition. The release of the reagent may enable contact of the reagent with the subdivided sample. For example, if the reagent is a unique identifier such as a barcode, the sample may be tagged with the unique identifier. The tagged sample may then be used in a downstream application such as a sequencing reaction.

A variety of different reactions and/operations may occur within a device disclosed herein, including but not limited to: sample partitioning, sample isolation, binding reactions, fragmentation (e.g., prior to partitioning or following partitioning), ligation reactions, and other enzymatic reactions. The device also may be useful for a variety of different molecular biology applications including, but not limited to, nucleic acid sequencing, protein sequencing, nucleic acid quantification, sequencing optimization, detecting gene expression, quantifying gene expression, and single-cell analysis of genomic or expressed markers. Moreover, the device has numerous medical applications. For example, it may be used for the identification, detection, diagnosis, treatment, staging of, or risk prediction of various genetic and non-genetic diseases and disorders including cancer.

II. Microcapsules

FIG. **1**A is a schematic of an exemplary microcapsule comprising an internal compartment **120** enveloped by a second layer **130**, which is encapsulated by a solid or semi-permeable shell or membrane **110**. In general, the shell separates the internal compartment(s) from their immediate environment (e.g., interior of a microwell). The internal compartments, e.g., **120**, **130**, may comprise materials such as reagents. As depicted in FIG. **1**A, the reagents **100** may be present in the internal compartment **120**. However, in some cases, the reagents are located in the enveloping layer **130** or in both compartments. Generally, the microcapsule may release the inner materials, or a portion thereof, following the introduction of a particular trigger. The trigger may cause disruption of the shell layer **110** and/or the internal enveloping layer **130**, thereby permitting contact of the internal compartment **100**, **120** with the outside environment, such as the cavity of a microwell.

The microcapsule may comprise several fluidic phases and may comprise an emulsion (e.g. water-in-oil emulsion, oil-in-water emulsion). A microcapsule may comprise an internal layer **120** that is immiscible with a second layer **130** enveloping the internal layer. For example, the internal layer **120** may comprise an aqueous fluid, while the enveloping layer **130** may be a non-aqueous fluid such as an oil. Conversely, the internal layer **120** may comprise a non-aqueous fluid (e.g., oil), and the enveloping layer **130** may comprise an aqueous fluid. In some cases, the microcapsule does not comprise an enveloping second layer. Often, the microcapsule is further encapsulated by a shell layer **110**, which may comprise a polymeric material. In some cases, a microcapsule may comprise a droplet.

Droplets and methods for droplet generation, for example, are described in U.S. Pat. No. RE41,780, which is incorporated herein by reference in its entirety for all purposes. The device also may contain a microfluidic element that enables the flow of a sample and/or microcapsules through the device and distribution of the sample and/or microcapsules within the partitions.

The microcapsule can comprise multiple compartments. The microcapsule may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 compartments. In other cases, the microcapsule comprises less than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 compartments. Similarly, each compartment, or a subset thereof, may also be subdivided into a plurality of additional compartments. In some cases, each compartment, or subset thereof, is subdivided into at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 compartments. In other cases, each compartment, or subset thereof, is further subdivided into less than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 compartments.

There are several possible distributions of reagent in the multiple compartments. For example, each compartment (or some percentage of the total number of compartments) may comprise the same reagent or the same combination or reagents. In some cases, each compartment (or some percentage of the total number of compartments) comprises different reagents or a different combination of reagents.

The compartments may be configured in a variety of ways. In some cases, the microcapsule may comprise multiple concentric compartments (repeating units of compartments that contain the preceding compartment), often separated by an immiscible layer. In such microcapsules, the reagents may be present in alternating compartments, in every third compartment, or in every fourth compartment.

In some cases, most of the compartments with a microcapsule are not concentric; instead, they exist as separate, self-contained entities within a microcapsule. FIG. **1**B depicts an example of a microcapsule that contains a plurality of smaller microcapsules **140**, each containing a reagent. Like many of the other microcapsules described herein, the microcapsule may be encapsulated by an outer shell, often comprising a polymer material **150**. The plurality of smaller microcapsules encapsulated within the larger microcapsule may be physically separated by an immiscible fluid **160**, thereby preventing mixing of reagents before application of a stimulus and release of reagents into solution. In some cases, the immiscible fluid is loaded with additional materials or reagents. In some cases, the plurality of smaller microcapsules are surrounded by a layer of immiscible fluid (e.g., **170**) which is further surrounded by a fluid **160** that is miscible with the inner fluid of the microcapsules. For example, the interior microcapsules **180** may comprise an aqueous interior enveloped by an immiscible (e.g., oil) layer, that is further surrounded by an aqueous layer **160**. The miscible compartments (e.g., **160** and **180**) may each contain reagents. They may contain the same reagents (or the same combination of reagents) or different reagents (or different combination of reagents). Alternatively, one or some of the miscible compartments may comprise no reagents.

The microcapsule may comprise a polymeric shell (see, e.g., FIGS. **1** and **2**) or multiple polymeric shells. For example, the microcapsule may comprise multiple polymeric shells layered on top of each other. In other cases, individual compartments within a microcapsule comprise a polymeric shell, or a subset of the compartments may comprise a polymeric shell. For example, all or some of the smaller compartments **140** in FIG. **1**B may comprise a polymeric shell that separates them from the fluidic interior **160**. The microcapsule may be designed so that a particular reagent is contained within a compartment that has a polymerized shell, while a different reagent is within a compartment that is simply enveloped by an immiscible liquid. For example, a reagent that is desired to be released upon a heat trigger may be contained within the compartments that have a heat-sensitive or heat-activatable polymerized shell, while reagents designed to be released upon a different trigger may be present in different types of compartments. In another example, paramagnetic particles may be incorporated into the capsule shell wall. A magnet or electric field may then be used to position the capsule to a desired location. In some cases, a magnetic field (e.g., high frequency alternating magnetic field) can be applied to such capsules; the incorporated paramagnetic particles may then transform the energy of the magnetic field into heat, thereby triggering rupture of the capsule.

The microcapsule component of a device of this disclosure may provide for the controlled and/or timed release of reagents for sample preparation of an analyte. Microcapsules may be used in particular for controlled release and transport of varying types of chemicals, ingredients, pharmaceuticals, fragrances etc. . . . , including particularly

sensitive reagents such as enzymes and proteins (see, e.g., D. D. Lewis, "Biodegradable Polymers and Drug Delivery Systems", M. Chasin and R. Langer, editors (Marcel Decker, New York, 1990); J. P. McGee et al., J. Control. Release 34 (1995), 77).

Microcapsules may also provide a means for delivery of reagents in discrete and definable amounts. Microcapsules may be used to prevent premature mixing of reagents with the sample, by segregating the reagents from the sample. Microcapsules also may ease handling of—and limit contacts with—particularly sensitive reagents such as enzymes, nucleic acids and other chemicals used in sample preparation.

A. Preparation of Microcapsules

Microcapsules of a device of this disclosure may be prepared by numerous methods and processes. Preparative techniques may include pan coating, spray drying, centrifugal extrusion, emulsion-based methods, and/or microfluidic techniques. Typically, a method for preparation is chosen based on the desired characteristics of the microcapsule. For example, shell wall thickness, permeability, chemical composition of the shell wall, mechanical integrity of the shell wall and capsule size may be taken into consideration when choosing a method. Methods of preparation may also be selected based on the ability to incorporate specific materials within the capsule such as whether the core materials (e.g., fluids, reagents, etc.) are aqueous, organic or inorganic. Additionally, preparation methods can affect the shape and size of the microcapsule. For example a capsule's shape, (e.g., spherical, ellipsoidal, etc.), may depend on the shape of the droplet in the precursor liquid which may be determined by the viscosity and surface tension of the core liquid, direction of flow of the emulsion, the choice of surfactants used in droplet stabilization, as well as physical confinement such as preparations made in a microchannel or capillary of a particular size (e.g., a size requiring distortion of the microcapsule in order for the microcapsule to fit within the microchannel or capillary.

Microcapsules may be prepared through emulsification polymerization, a process in which monomer units at an aqueous/organic interface in an emulsion polymerize to form a shell. Reagents are mixed with the aqueous phase of the biphasic mixture. Vigorous shaking, or sonication of the mixture, creates droplets containing reagents, which are encased by a polymeric shell.

In some cases, microcapsules may be prepared through layer-by-layer assembly, a process in which negatively and positively charged polyelectrolytes are deposited onto particles such as metal oxide cores. Electrostatic interactions between polyelectrolytes create a polymeric shell around the core. The core can be subsequently removed via addition of acid, resulting in a semi-permeable hollow sphere which can be loaded with various reagents.

In still further cases, microcapsules may be prepared through coacervation, a process in which two oppositely charged polymers in aqueous solution become entangled to form a neutralized polymer shell wall. One polymer may be contained within an oil phase, while the other, of opposite charge is contained in an aqueous phase. This aqueous phase may contain reagents to be encapsulated. The attraction of one polymer for another can result in the formation of coascervates. In some embodiments, gelatin and gum Arabic are components of this preparative method.

Microcapsules also may be prepared through internal phase separation, a process in which a polymer is dissolved in a solvent mixture containing volatile and nonvolatile solvents. Droplets of the resultant solution are suspended in an aqueous layer, which is stabilized by continual agitation and the use of surfactants. This phase may contain reagents to be encapsulated. When the volatile solvent evaporates, the polymers coalesce to form a shell wall. In some cases, polymers such as polystyrene, poly(methyl methacrylate) and poly(tetrahydrofuran) are used to form shell walls.

Microcapsules also may be prepared through flow focusing methods, a process in which a microcapillary device is used to generate double emulsions containing a single internal droplet encased in a middle fluid which is then dispersed to an outer fluid. The inner droplet may contain reagents to be encapsulated. The middle fluid becomes the shell wall, which can be formed via cross-linking reactions.

B. Microcapsule Composition

Microcapsules may comprise a variety of materials with a wide range of chemical characteristics. Generally, the microcapsules comprise materials with the ability to form microcapsules of a desired shape and size and that are compatible with the reagents to be stored in the microcapsules.

Microcapsules may comprise a wide range of different polymers including but not limited to: polymers, heat sensitive polymers, photosensitive polymers, magnetic polymers, pH sensitive polymers, salt-sensitive polymers, chemically sensitive polymers, polyelectrolytes, polysaccharides, peptides, proteins, and/or plastics. Polymers may include but are not limited to materials such as poly(N-isopropylacrylamide) (PNIPAAm), poly(styrene sulfonate) (PSS), poly(allyl amine) (PAAm), poly(acrylic acid) (PAA), poly(ethylene imine) (PEI), poly(diallyldimethyl-ammonium chloride) (PDADMAC), poly(pyrolle) (PPy), poly (vinylpyrrolidone) (PVPON), poly(vinyl pyridine) (PVP), poly(methacrylic acid) (PMAA), poly(methyl methacrylate) (PMMA), polystyrene (PS), poly(tetrahydrofuran) (PTHF), poly(phthaladehyde) (PTHF), poly(hexyl viologen) (PHV), poly(L-lysine) (PLL), poly(L-arginine) (PARG), poly(lactic-co-glycolic acid) (PLGA).

Often, materials for the microcapsules, particularly the shells of microcapsules, may enable the microcapsule to be disrupted with an applied stimulus. For example, a microcapsule may be prepared from heat sensitive polymers and/or may comprise one or more shells comprising such heat-sensitive polymers. The heat-sensitive polymer may be stable under conditions used for storage or loading. Upon exposure to heat, the heat-sensitive polymer components may undergo depolymerization, resulting in disruption to the integrity of the shell and release of the inner materials of the microcapsule (and/or of the inner microcapsules) to the outside environment (e.g., the interior of a microwell). Exemplary heat-sensitive polymers may include, but are not limited to NIPAAm or PNIPAM hydrogel. The microcapsules may also comprise one or more types of oil. Exemplary oils include but are not limited to hydrocarbon oils, fluorinated oils, fluorocarbon oils, silicone oils, mineral oils, vegetable oils, and any other suitable oil.

The microcapsules may also comprise a surfactant, such as an emulsifying surfactant. Exemplary surfactants include, but are not limited to, cationic surfactants, non-ionic surfactants, anionic surfactants, hydrocarbon surfactants or fluorosurfactants. The surfactant may increase the stability of one or more components of the microcapsule, such as an inner compartment that comprises an oil.

Additionally, the microcapsules may comprise an inner material that is miscible with materials external to the capsule. For example, the inner material may be an aqueous fluid and the sample within the microwell may also be in an aqueous fluid. In other examples, the microcapsule may

comprise powders or nanoparticles that are miscible with an aqueous fluid. For example, the microcapsule may comprise such powders or nanoparticles in an inner compartment. Upon disruption of the microcapsule, such powders or nanoparticles are released into the external environment (e.g., interior of microwell) and may mix with an aqueous fluid (e.g., an aqueous sample fluid).

Additionally, the microcapsule may comprise a material that is immiscible with the surrounding environment (e.g., interior of microwell, sample fluid). In such cases, when the inner emulsion is released to the surrounding environment, the phase separation between the inner and outer components may promote mixing, such as mixing of the inner components with the surrounding fluid. In some cases, when a microcapsule is triggered to release its contents, a pressure or force is also released that promotes mixing of internal and external components.

The microcapsules may also comprise a polymer within the interior of the capsule. In some instances this polymer may be a porous polymer bead that may entrap reagents or combinations of reagents. In other instances, this polymer may be a bead that has been previously swollen to create a gel. Examples of polymer based gels that may be used as inner emulsions of capsules may include, but are not limited to sodium alginate gel, or poly acrylamide gel swelled with oligonucleotide bar codes or the like.

In some cases, a microcapsule may be a gel bead comprising any of the polymer based gels described herein. Gel bead microcapsules may be generated, for example, by encapsulating one or more polymeric precursors into droplets. Upon exposure of the polymeric precursors to an accelerator (e.g., tetramethylethylenediamine (TEMED)), a gel bead may be generated.

Analytes and/or reagents, such as oligonucleotide barcodes, for example, may be coupled/immobilized to the interior surface of a gel bead (e.g., the interior accessible via diffusion of an oligonucleotide barcode and/or materials used to generate an oligonucleotide barcode) and/or the outer surface of a gel bead or any other microcapsule described herein. Coupling/immobilization may be via any form of chemical bonding (e.g., covalent bond, ionic bond) or physical phenomena (e.g., Van der Waals forces, dipole-dipole interactions, etc.). In some cases, coupling/immobilization of a reagent to a gel bead or any other microcapsule described herein may be reversible, such as, for example, via a labile moiety (e.g., via a chemical cross-linker, including chemical cross-linkers described herein). Upon application of a stimulus, the labile moiety may be cleaved and the immobilized reagent set free. In some cases, the labile moiety is a disulfide bond. For example, in the case where an oligonucleotide barcode is immobilized to a gel bead via a disulfide bond, exposure of the disulfide bond to a reducing agent can cleave the disulfide bond and free the oligonucleotide barcode from the bead. The labile moiety may be included as part of a gel bead or microcapsule, as part of a chemical linker that links a reagent or analyte to a gel bead or microcapsule, and/or as part of a reagent or analyte.

A gel bead or any other type of microcapsule described herein may contain varied numbers of reagents. The density of a reagent per microcapsule may vary depending on the particular microcapsule utilized and the particular reagent. For example, a microcapsule or gel bead may comprise at least about 1; 10; 100; 1,000; 10,000; 100,000; 1,000,000; 5,000,000; 10,000,000, 50,000,000; 100,000,000; 500,000,000; or 1,000,000,000 oligonucleotide barcodes per microcapsule or gel bead. A gel bead may comprise identical oligonucleotide barcodes or may comprise differing oligonucleotide barcodes.

In other example, the microcapsule may comprise one or more materials that create a net neutral, negative or positive charge on the outer shell wall of the capsule. In some instances, the charge of a capsule may aid in preventing or promoting aggregation or clustering of particles, or adherence or repulsion to parts of the device.

In addition, the microcapsule may comprise one or more materials that cause the outer shell wall of the capsule to be hydrophilic or hydrophobic. A hydrophilic material that may be used for capsule shell walls may be poly(N-isopropylacrylamide). A hydrophobic material that may be used for capsule shell walls may be polystyrene. In certain instances, a hydrophilic shell wall may aid in wicking of the capsule into wells comprising aqueous fluid.

C. Microcapsule Size and Shape

A microcapsule may be any of a number of sizes or shapes. In some cases, the shape of the microcapsule may be spherical, ellipsoidal, cylindrical, hexagonal or any other symmetrical or non-symmetrical shape. Any cross-section of the microcapsule may also be of any appropriate shape, include but not limited to: circular, oblong, square, rectangular, hexagonal, or other symmetrical or non-symmetrical shape. In some cases, the microcapsule may be of a specific shape that complements an opening (e.g., surface of a microwell) of the device. For example, the microcapsule may be spherical and the opening of a microwell of the device may be circular.

The microcapsules may be of uniform size (e.g., all of the microcapsules are the same size) or heterogeneous size (e.g., some of the microcapsules are of different sizes). A dimension (e.g., diameter, cross-section, side, etc.) of a microcapsule may be at least about 0.001 μm, 0.01 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm or 1 nm. In some cases, the microcapsule comprises a microwell that is at most about 0.001 μm, 0.01 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm or 1 nm.

In some cases, microcapsules are of a size and/or shape so as to allow a limited number of microcapsules to be deposited in individual partitions (e.g., microwells, droplets) of the microcapsule array. Microcapsules may have a specific size and/or shape such that exactly or no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 capsules fit into an individual microwell; in some cases, on average 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 capsules fit into an individual microwell. In still further cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 100, 500, or 1000 capsules fit into an individual microwell.

D. Reagents and Reagent Loading

The devices provided herein may comprise free reagents and/or reagents encapsulated into microcapsules. The reagents may be a variety of molecules, chemicals, particles, and elements suitable for sample preparation reactions of an analyte. For example, a microcapsule used in a sample preparation reaction for DNA sequencing of a target may comprise one or more of the following reagents: enzymes, restriction enzymes (e.g., multiple cutters), ligase, polymerase (e.g., polymerases that do and do not recognize dUTPs and/or uracil), fluorophores, oligonucleotide barcodes, buffers, deoxynucleotide triphosphates (dNTPs) (e.g. deoxyadenosine triphosphate (dATP), deoxycitidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP), deoxyuridine triphosphate (dUTP)), deoxynucleotide triphosphates (ddNTPs) and the

like. In another example, a microcapsule used in a sample preparation reaction for single cell analysis may comprise reagents such as one or more of the following reagents: lysis buffer, detergent, fluorophores, oligonucleotide barcodes, ligase, proteases, heat activatable proteases, protease or nuclease inhibitors, buffer, enzymes, antibodies, nanoparticles, and the like.

Exemplary reagents include, but are not limited to: buffers, acidic solution, basic solution, temperature-sensitive enzymes, pH-sensitive enzymes, light-sensitive enzymes, metals, metal ions, magnesium chloride, sodium chloride, manganese, aqueous buffer, mild buffer, ionic buffer, inhibitor, enzyme, protein, nucleic acid, antibodies, saccharides, lipid, oil, salt, ion, detergents, ionic detergents, non-ionic detergents, oligonucleotides, nucleotides, dNTPs, ddNTPs, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), peptide nucleic acids, circular DNA (cDNA), double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), plasmid DNA, cosmid DNA, chromosomal DNA, genomic DNA (gDNA), viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), nRNA, short-interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajul body specific RNA, (scaRNA), microRNA, double-stranded RNA (dsRNA), ribozyme, riboswitch and viral RNA, polymerase (e.g., polymerases that do and do not recognize dUTPs and/or uracil), ligase, restriction enzymes, proteases, nucleases, protease inhibitors, nuclease inhibitors, chelating agents, reducing agents (e.g., dithiotheritol (DTT), 2-tris(2-carboxyethyl) phosphine (TCEP)), oxidizing agents, fluorophores, probes, chromophores, dyes, organics, emulsifiers, surfactants, stabilizers, polymers, water, small molecules, pharmaceuticals, radioactive molecules, preservatives, antibiotics, aptamers, and pharmaceutical drug compounds.

In some cases, a microcapsule comprises a set of reagents that have a similar attribute (e.g., a set of enzymes, a set of minerals, a set of oligonucleotides, a mixture of different bar-codes, a mixture of identical bar-codes). In other cases, a microcapsule comprises a heterogeneous mixture of reagents. In some cases, the heterogeneous mixture of reagents comprises all components necessary to perform a reaction. In some cases, such mixture comprises all components necessary to perform a reaction, except for 1, 2, 3, 4, 5, or more components necessary to perform a reaction. In some cases, such additional components are contained within a different microcapsule or within a solution within a partition (e.g., microwell) of the device.

Reagents may be pre-loaded into the device (e.g., prior to introduction of analyte) or post-loaded into the device. They may be loaded directly into the device; or, in some cases, the reagents are encapsulated into a microcapsule that is loaded into the device. In some cases, only microcapsules comprising reagents are introduced. In other cases, both free reagents and reagents encapsulated in microcapsules are loaded into the device, either sequentially or concurrently. In some cases, reagents are introduced to the device either before or after a particular step. For example, a lysis buffer reagent may be introduced to the device following partitioning of a cellular sample into multiple partitions (e.g., microwells, droplets) within the device. In some cases, reagents and/or microcapsules comprising reagents are introduced sequentially such that different reactions or operations occur at different steps. The reagents (or microcapsules) may be also be loaded at steps interspersed with a reaction or operation step. For example, microcapsules comprising reagents for fragmenting molecules (e.g., nucleic acids) may be loaded into the device, followed by a fragmentation step, which may be followed by loading of microcapsules comprising reagents for ligating bar-codes (or other unique identifiers, e.g., antibodies) and subsequent ligation of the bar-codes to the fragmented molecules. Additional methods of loading reagents are described further herein in other sections.

E. Molecular 'Barcodes'

It may be desirable to retain the option of identifying and tracking individual molecules or analytes after or during sample preparation. In some cases, one or more unique molecular identifiers, sometimes known in the art as a 'molecular barcodes,' are used as sample preparation reagents. These molecules may comprise a variety of different forms such as oligonucleotide bar codes, antibodies or antibody fragments, fluorophores, nanoparticles, and other elements or combinations thereof. Depending upon the specific application, molecular barcodes may reversibly or irreversibly bind to the target analyte and allow for identification and/or quantification of individual analytes after recovery from a device after sample preparation.

A device of this disclosure may be applicable to nucleic acid sequencing, protein detection, single molecule analysis and other methods that require a) precise measurement of the presence and amount of a specific analyte b) multiplex reactions in which multiple analytes are pooled for analysis. A device of this disclosure may utilize the microwells of the microwell array or other type of partition (e.g., droplets) to physically partition target analytes. This physical partitioning allows for individual analytes to acquire one or more molecular barcodes. After sample preparation, individual analytes may be pooled or combined and extracted from a device for multiplex analysis. For most applications, multiplex analysis substantially decreases the cost of analysis as well as increases through-put of the process, such as in the case of the nucleic acid sequencing. Molecular barcodes may allow for the identification and quantification of individual molecules even after pooling of a plurality of analytes. For example, with respect to nucleic acid sequencing, molecular barcodes may permit the sequencing of individual nucleic acids, even after the pooling of a plurality of different nucleic acids.

Oligonucleotide barcodes, in some cases, may be particularly useful in nucleic acid sequencing. In general, an oligonucleotide barcode may comprise a unique sequence (e.g., a barcode sequence) that gives the oligonucleotide barcode its identifying functionality. The unique sequence may be random or non-random. Attachment of the barcode sequence to a nucleic acid of interest may associate the barcode sequence with the nucleic acid of interest. The barcode may then be used to identify the nucleic acid of interest during sequencing, even when other nucleic acids of interest (e.g., comprising different barcodes) are present. In cases where a nucleic acid of interest is fragmented prior to sequencing, an attached barcode may be used to identify fragments as belonging to the nucleic acid of interest during sequencing.

An oligonucleotide barcode may consist solely of a unique barcode sequence or may be included as part of an oligonucleotide of longer sequence length. Such an oligonucleotide may be an adaptor required for a particular sequencing chemistry and/or method. For example, such adaptors may include, in addition to an oligonucleotide barcode, immobilization sequence regions necessary to immobilize (e.g., via hybridization) the adaptor to a solid surface (e.g., solid surfaces in a sequencer flow cell channel); sequence regions required for the binding of sequenc-

ing primers; and/or a random sequence (e.g., a random N-mer) that may be useful, for example, in random amplification schemes. An adaptor can be attached to a nucleic acid to be sequenced, for example, by amplification, ligation, or any other method described herein.

Moreover, an oligonucleotide barcode, and/or a larger oligonucleotide comprising an oligonucleotide barcode may comprise natural nucleic acid bases and/or may comprise non-natural bases. For example, in cases where an oligonucleotide barcode or a larger oligonucleotide comprising an oligonucleotide barcode is DNA, the oligonucleotide may comprise the natural DNA bases adenine, guanine, cytosine, and thymine and/or may comprise non-natural bases such as uracil.

F. Microcapsule-Preparation for Microwell Loading

Following preparation, reagent loaded microcapsules may be loaded into a device using a variety of methods. Microcapsules, in some instances, may be loaded as 'dry capsules.' After preparation, capsules may be separated from a liquid phase using various techniques, including but not limited to differential centrifugation, evaporation of the liquid phase, chromatography, filtration and the like. 'Dry capsules' may be collected as a powder or particulate matter and then deposited into microwells of the microwell array. Loading 'dry capsules' may be a preferred method in instances in which loading of 'wet capsules,' leads to inefficiencies of loading such as empty wells and poor distribution of microcapsules across the microwell array.

Reagent-loaded microcapsules may also be loaded into a device when the microcapsules are within a liquid phase, and thereby loaded as 'wet capsules.' In some instances, microcapsules may be suspended in a volatile oil such that the oil can be removed or evaporated, leaving only the dry capsule in the well. Loading 'wet capsules' may be a preferred method in some instances in which loading of dry capsules leads to inefficiencies of loading, such as microcapsule clustering, aggregation and poor distribution of microcapsules across the microwell array. Additional methods of loading reagents and microcapsules are described in other sections of this disclosure.

The microcapsules also may have a particular density. In some cases, the microcapsules are less dense than an aqueous fluid (e.g., water); in some cases, the microcapsules are denser than an aqueous fluid (e.g., water). In some cases, the microcapsules are less dense than a non-aqueous fluid (e.g., oil); in some cases, the microcapsules are denser than a non-aqueous fluid (e.g., oil). Microcapsules may comprise a density at least about 0.05 g/cm^3, 0.1 cm^3, 0.2 g/cm^3, 0.3 g/cm^3, 0.4 g/cm^3, 0.5 g/cm^3, 0.6 g/cm^3, 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. In other cases, microcapsule densities may be at most about 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. Such densities can reflect the density of the microcapsule in any particular fluid (e.g., aqueous, water, oil, etc.)

III. Microwell Array

A. Structure/Features

A device of this disclosure may be a microwell array comprising a solid plate containing a plurality of holes, cavities or microwells in which microcapsules and/or analytes are deposited. Generally, a fluidic sample (or analyte) is introduced into the device (e.g., through an inlet) and then travels through a flow channel which distributes the sample into multiple microwells. In some cases, additional fluid is introduced into the device as well. The microwells may comprise microcapsules when the sample is introduced; or, in some cases, the microcapsules are introduced into the microwells following introduction of the sample.

FIG. **2**A depicts a prototype microwell array; a sideview is depicted in FIG. **2**B. The microwell array may include a plate **220** that can be made of any suitable material commonly used in a chemical laboratory, including fused silica, soda lima glass, borosilicate glass, PMMA, sapphire, silicon, germanium, cyclic olefin copolymer and cyclic polymer, polyethylenes, polypropylenes, polyacrylates, polycarbonates, plastics, Topas, and other suitable substrates known in the art. The plate **220** may initially be a flat solid plate comprising a regular pattern of microwells **270**. The microwells may be formed by drilling or chemical dissolution or any other suitable method of machining; however, plates with a desired hole pattern are preferably molded, e.g. by injection-molding, embossing, or using a suitable polymer, such as cyclic olefin copolymer.

The microwell array may comprise an inlet (**200** and **240**) and/or an outlet (**210** and **260**); in some cases, the microwell array comprises multiple inlets and/or outlets. A sample (or analyte) or microcapsules may be introduced to the device via the inlet. Solutions containing analytes, reagents and/or microcapsules may be manually applied to the inlet port **200** and **240** (or to a conduit attached to the inlet port) via a pipette. In some cases, a liquid handling device is used to introduce analytes, reagents, and/or microcapsules to the device. Exemplary liquid handling devices may rely on a pipetting robot, capillary action, or dipping into a fluid. In some cases, the inlet port is connected to a reservoir comprising microcapsules or analytes. The inlet port may be attached to a flow channel **250** that permits distribution of the analyte, sample, or microcapsules to the microwells in the device. In some cases, the inlet port may be used to introduce to the device a fluid (e.g., oil, aqueous) that does not contain microcapsules or analyte, such as a carrier fluid. The carrier fluid may be introduced via the inlet port before, during, or following the introduction of analyte and/or microcapsules. In cases where the device has multiple inlets, the same sample may be introduced via the multiple inlets, or each inlet may convey a different sample. In some cases, one inlet may convey a sample or analyte to the microwells, while a different inlet conveys free reagents and/or reagents encapsulated in microcapsules to the device. The device may have at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 inlets and/or outlets.

In some cases, solutions containing microcapsules and/or analytes may be pulled through the device via a vacuum manifold attached to the outlet port **210** and **260**. Such manifold may apply a negative pressure to the device. In other cases, a positive pressure is used to move sample, analytes, and/or microcapsules through the device. The area, length, and width of surfaces of **230** according to this disclosure may be varied according to the requirements of the assay to be performed. Considerations may include, for example, ease of handling, limitations of the material(s) of which the surface is formed, requirements of detection or processing systems, requirements of deposition systems

(e.g. microfluidic systems), and the like. The thickness may comprise a thickness of at least about 0.001 mm, 0.005 mm, 0.01 mm, 0.05 mm, 0.1 mm, 0.2 mm, 0.3 mm, 0.4 mm, 0.5 mm, 0.6 mm, 0.7 mm, 0.8 mm, 0.9 mm, 1.0 mm, 2.0 mm, 3.0 mm, 4.0 mm, 5.0 mm, 6.0 mm, 7.0 mm, 8.0 mm, 9.0 mm, 10.0 mm, 11 mm, 12 mm, 13 mm, 14 mm, or 15 mm. In other cases, microcapsule thickness may be at most 0.001 mm, 0.005 mm, 0.01 mm, 0.05 mm, 0.1 mm, 0.2 mm, 0.3 mm, 0.4 mm, 0.5 mm, 0.6 mm, 0.7 mm, 0.8 mm, 0.9 mm, 1.0 mm, 2.0 mm, 3.0 mm, 4.0 mm, 5.0 mm, 6.0 mm, 7.0 mm, 8.0 mm, 9.0 mm, 10.0 mm, 11 mm, 12 mm, 13 mm, 14 mm, or 15 mm.

The microwells **270** can be any shape and size suitable for the assay performed. The cross-section of the microwells may have a cross-sectional dimension that is circular, rectangular, square, hexagonal, or other symmetric or non-symmetric shape. In some cases, the shape of the microwell may be cylindrical, cubic, conical, frustoconical, hexagonal or other symmetric or non-symmetric shape. The diameter of the microwells **270** may be determined by the size of the wells desired and the available surface area of the plate itself. Exemplary microwells comprise diameters of at least 0.01 μm, 0.1 μm, 0.2 μm, 0.3 μm, 0.4 μm, 0.5 μm, 1 μm, 10 μm, 25 μm, 50 μm, 75 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, 1.0 mm. In other cases, microwell diameters may comprise at most 0.01 μm, 0.1 μm, 0.2 μm, 0.3 μm, 0.4 μm, 0.5 μm, 1 μm, 10 μm, 25 μm, 50 μm, 75 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm or 1.0 mm.

The capacity (or volume) of each well can be a measure of the height of the well (the thickness of the plate) and the effective diameter of each well. The capacity of an individual well may be selected from a wide range of volumes. In some cases, the device may comprise a well (or microwell) with a capacity of at least 0.001 fL, 0.01 fL, 0.1 fL, 0.5 fL, 1 fL, 5 fL, 10 fL, 50 fL, 100 fL, 200 fL, 300 fL, 400 fL, 500 fL, 600 fL, 700 fL, 800 fL, 900 fL, 1 pL, 5 pL, 10 pL, 50 pL, 100 pL, 200 pL, 300 pL, 400 pL, 500 pL, 600 pL, 700 pL, 800 pL, 900 pL, 1 nL, 5 nL, 10 nL, 50 nL, 100 nL, 200 nL, 300 nL, 400 nL, 500 nL, 1 uL, 50 uL, or 100 uL. In other cases, the microcapsule comprises a microwell that is less than 0.001 fL, 0.01 fL, 0.1 fL, 0.5 L, 5 fL, 10 fL, 50 fL, 100 fL, 200 fL, 300 fL, 400 fL, 500 fL, 600 fL, 700 fL, 800 fL, 900 fL, 1 pL, 5 pL, 10 pL, 50 pL, 100 pL, 200 pL, 300 pL, 400 pL, 500 pL, 600 pL, 700 pL, 800 pL, 900 pL, 1 nL, 5 nL, 10 nL, 50 nL, 100 nL, 200 nL, 300 nL, 400 nL, 500 nL, 1 uL, 50 uL, or 100 uL.

There may be variability in the volume of fluid in different microwells in the array. More specifically, the volume of different microwells may vary by at least (or at most) plus or minus 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, or 1000% across a set of microwells. For example, a microwell may comprise a volume of fluid that is at most 80% of the fluid volume within a second microwell.

Based on the dimension of individual microwells and the size of the plate, the microwell array may comprise a range of well densities. In some examples, a plurality of microwells may have a density of at least about 2,500 wells/cm^2, at least about 1,000 wells/cm^2. In some cases, the plurality of wells may have a density of at least 10 wells/cm^2. In other cases, the well density may comprise at least 10 wells/cm^2, 50 wells/cm^2, 100 wells/cm^2, 500 wells/cm^2, 1000 wells/cm^2, 5000 wells/cm^2, 10000 wells/cm^2, 50000 wells/cm^2, or 100000 wells/cm^2. In other cases, the well density may be less than 100000 wells/cm^2, 10000 wells/cm^2, 5000 wells/cm^2, 1000 wells/cm^2, 500 wells/cm^2, or 100 wells/cm^2.

In some cases, the interior surface of the microwells comprises a hydrophilic material that preferably accommodates an aqueous sample; in some cases, the region between the microwells is composed of a hydrophobic material that may preferentially attract a hydrophobic sealing fluid described herein.

Multiple microwell arrays, e.g., FIG. **2**B may be arranged within a single device. FIG. **3**, **300**. For example, discrete microwell array slides may be arrayed in parallel on a plate holder. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 25, 50 or 100 microwell arrays are arrayed in parallel. In other cases, at most 100, 50, 25, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 devices are arrayed in parallel. The microwell arrays within a common device may be manipulated simultaneously or sequentially. For example, arrayed devices may be loaded with samples or capsules simultaneously or sequentially.

B. Microwell Array Fluids

The microwell array may comprise any of a number of different fluids including aqueous, non-aqueous, oils, and organic solvents, such as alcohols. In some cases, the fluid is used to carry a component, e.g., reagent, microcapsule, or analyte, to a target location such as microwells, output port, etc. In other cases, the fluid is used to flush the system. In still other cases, the fluid may be used to seal the microwells.

Any fluid or buffer that is physiologically compatible with the analytes (e.g., cells, molecules) or reagents used in the device may be used. In some cases, the fluid is aqueous (buffered or not buffered). For example, a sample comprising a population of cells suspended in a buffered aqueous solution may be introduced into the microwell array, allowed to flow through the device, and distributed to the microwells. In other cases, the fluid passing through the device is nonaqueous (e.g., oil). Exemplary non-aqueous fluids include but are not limited to: oils, non-polar solvent, hydrocarbon oil, decane (e.g., tetradecane or hexadecane), fluorocarbon oil, fluorinated oil, silicone oil, mineral oil, or other oil.

Often, the microcapsules are suspended in a fluid that is compatible with the components of the shell of the microcapsule. Fluids including but not limited to water, alcohols, hydrocarbon oils or fluorocarbon oils are particularly useful fluids for suspending and flowing microcapsules through the microarray device.

C. Further Partitioning and Sealing

After the analyte, free reagents, and/or microcapsules are loaded into the device and distributed to the microwells, a sealing fluid may be used to further partition or isolate them within the microwells. The sealing fluid may also be used to seal the individual wells. The sealing fluid may be introduced through the same inlet port that was used to introduce the analyte, reagents and/or microcapsules. But in some cases, the sealing fluid is introduced to the device by a separate inlet port, or through multiple separate inlet ports.

Often, the sealing fluid is a non-aqueous fluid (e.g., oil). When the sealing fluid flows through the microwell array device, it may displace excess aqueous solution (e.g., solution comprising analytes, free reagents and/or microcapsules) from individual microwells, thereby potentially removing aqueous bridges between adjacent microwells. The wells themselves, as described herein, may comprise a hydrophilic material that enables wicking of the aqueous fluids (e.g., sample fluid, microcapsule fluid) into individual wells. In some cases, regions external to the wells comprise hydrophobic material, again to encourage the positioning of the aqueous fluid into the interior of the microwells.

The sealing fluid may either remain in the device or be removed. The sealing fluid may be removed, e.g., by flowing

through the outlet port. In other cases, the sealing oil may comprise a volatile oil that can be removed by the application of heat. Once the sealing fluid is removed, analytes, free reagents and/or microcapsules may be physically partitioned from one another in the microwells.

A fluid may be selected such that its density is equal to, greater than or less than the density of the microcapsules. For example, the microcapsules may be denser than the sealing oil and/or aqueous fluid of the sample and reagents, thereby enabling the microcapsules to remain in the microwells as the sealing oil flows through the device. In another example, the capsules may be less dense than the aqueous fluid of the sample or the fluid that the microcapsules are suspended in, as described herein, thereby facilitating movement and distribution of the capsules across the plurality of microwells in a device.

In the case of microcapsules comprising paramagnetic material, a magnetic field may be used to load or direct the capsules into the microwells. A magnetic field may also be used to retain such microcapsules within the wells while the wells are being filled with sample, reagent, and/or sealing fluids. The magnetic field may also be used to remove capsule shells from the wells, particularly following rupture of the capsules.

In some cases, the sealing fluid may remain in the microwells when operations or reactions are conducted therein. The presence of the sealing fluid may act to further partition, isolate, or seal the individual microwells. In other cases, the sealing fluid may act as a carrier for the microcapsules. For example, sealing fluid comprising microcapsules may be introduced to the device to facilitate distribution of the microcapsules to the individual microwells. For such applications, the sealing fluid may be denser than the microcapsules in order to encourage more even distribution of the microcapsules to the microwells. Upon application of a stimulus, the microcapsules within the sealing fluid may release reagents to the microwell. In some cases, the sealing fluid may comprise a chemical or other agent capable of traveling from the sealing fluid to a well (e.g., by leaching or other mechanism) and triggering capsule rupture, where the capsule is present within the microwell or within the sealing fluid.

Methods other than those involving sealing fluids may also be used to seal the microwells following the loading of the analyte, free reagents, and/or microcapsules. For example, the microwells may be sealed with a laminate, tape, plastic cover, oils, waxes, or other suitable material to create an enclosed reaction chamber. The sealants described herein may protect the contents of the microwells from evaporation or other unintended consequences of the reactions or operations. Prevention of evaporation may be particularly necessary when heat is applied to the device, e.g., when heat is applied to stimulate microcapsule release.

In some cases, the laminate seal may also allow recovery of contents from individual wells. In this case, a single well of interest may be unsealed (e.g., by removal of the laminate seal) at a given time in order to enable further analysis of an analyte such as by MALDI mass spectrometry. Such applications may be useful in a number of settings, including high-throughput drug screening.

III. Loading Step(s)

As described herein, analytes, free reagents, and/or microcapsules may be loaded into the present device in any appropriate manner or order. The loading may be random or non-random. In some cases, a precise number of analytes and/or microcapsules are loaded into each individual microwell. In some cases, a precise number of analytes and/or microcapsules are loaded into a particular subset of microwells in the plate. In still other cases, an average number of analytes and/or micrcocapsules are loaded into each individual microwell. Furthermore, as described herein, in some cases, "dry" microcapsules are loaded into the device, while in other cases "wet" microcapsules are loaded into the device. In some cases, a combination of "dry" and "wet" microcapsules and/or reagents are loaded into the device, either simultaneously or sequentially.

As mentioned herein, the loading of the device may occur in any order and may occur in multiple stages. In some cases, the microcapsules are pre-loaded into the device, prior to the loading of the analyte. In other cases, the microcapsules and analyte are loaded concurrently. In still other cases, the analytes are loaded before the microcapsules are loaded.

The microcapsules and/or analytes may be loaded in multiple stages or multiple times. For example, microcapsules may be loaded into the device both prior to and after analytes are loaded into the device. The microcapsules that are pre-loaded (e.g., loaded prior to the analyte introduction) may comprise the same reagents as the microcapsules loaded after the analyte introduction. In other cases, the pre-loaded microcapsules contain reagents that are different from the reagents within the microcapsules loaded after analyte introduction. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 different sets of microcapsules are loaded onto the device. In some cases, the different sets of microcapsules are loaded sequentially; or, different sets of microcapsules may also be loaded simultaneously. Similarly, multiple sets of analytes can be loaded into the device. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 different sets of analytes are loaded onto the device. In some cases, the different sets of analytes are loaded sequentially; or, different sets of analytes may also be loaded simultaneously.

This disclosure provides devices comprising certain numbers of microcapsules and/or analytes loaded per well. In some cases, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, or 100 microcapsules and/or analytes are loaded into each individual microwell. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, or 100 microcapsules and/or analytes are loaded into each individual microwell. In some cases, on average, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, or 100 microcapsules and/or analytes are loaded into each individual microwell. In other cases, on average, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, or 100 microcapsules and/or analytes are loaded into each individual microwell. In some cases, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, or 100 microcapsules and/or analytes are loaded into each individual microwell.

Analytes and/or microcapsules may be applied in a quantity that allows a desired number of analytes to be deposited into an individual microwell. For example, terminal dilution of analytes, such as cells, may achieve the loading of one cell per one microwell or any desired number of analytes per microwell. In some cases, a Poisson distribution is used to direct or predict the final concentration of analytes or microcapsules per well.

The microcapsules may be loaded into the microarray device in a particular pattern. For example, certain sections of the device may comprise microcapsules containing a particular reagent (e.g., unique bar-code, enzyme, antibody, antibody subclass, etc.), while other sections of the device may comprise microcapsules containing a different reagent (e.g., a different bar-code, different enzyme, different antibody different antibody subclass, etc.). In some cases, the

microcapsules in one section of the array may contain control reagents. For example, they may contain positive controls that include a control analyte and necessary materials for a reaction. Or, in some cases, the microcapsules contain negative control reagents such as deactivated enzyme, or a synthetic oligonucleotide sequence that is resistant to fragmentation. In some cases, negative control reagents may control for the specificity of the sample preparation reaction etc. In other cases, the negative control microcapsules may comprise the same reagents present in other microcapsules except that the negative control microcapsule may lack a certain reagent (e.g., lysis buffer, polymerase, etc.).

The analytes/sample also may be loaded into the microarray device in a particular pattern. For example, certain sections of the device may comprise particular analytes, such as control analytes or analytes deriving from a particular source. This may be used in combination with specific loading of bar codes into known well locations. This feature may allow mapping of specific locations on the array to sequence data, thereby reducing the number of bar codes to be used for labeling reactions.

In cases where a partition is a droplet, an analyte and reagents may be combined within the droplet with the aid of a microfluidic device. For example, a droplet may be generated that comprises a gel bead (e.g., comprising an oligonucleotide barcode) a nucleic acid analyte, and any other desired reagents. The gel bead, nucleic acid analyte, and reagents in an aqueous phase may be combined at a junction of two or more channels of a microfluidic device. At a second junction of two or more channels of the microfluidic device, a droplet comprising the resulting mixture may be generated by contacting the aqueous mixture of reagents, gel bead, and nucleic acid analyte with an oil continuous phase.

IV. Microcapsule Stimuli

Various different stimuli may be used to trigger release of reagents from the microcapsules, or from internal compartments therein. In some cases, a microcapsule is degradable. Generally, the trigger may cause disruption or degradation of the shell or membrane enveloping the microcapsule, disruption or degradation of the interior of a microcapsule, and/or disruption or degradation of any chemical bonds that immobilize a reagent to the microcapsule. Exemplary triggers include but are not limited to: chemical triggers, bulk changes, biological triggers, light triggers, thermal triggers, magnetic triggers, and any combination thereof. See, e.g., Esser-Kahn et al., (2011) *Macromolecules* 44: 5539-5553; Wang et al., (2009) *ChemPhysChem* 10:2405-2409;

A. Chemical Stimuli and Bulk Changes

Numerous chemical triggers may be used to trigger the disruption or degradation of the microcapsules. Examples of these chemical changes may include, but are not limited to pH-mediated changes to the shell wall, disintegration of the shell wall via chemical cleavage of crosslink bonds, triggered depolymerization of the shell wall, and shell wall switching reactions. Bulk changes may also be used to trigger disruption of the microcapsules.

A change in pH of the solution, particularly a decrease in pH, may trigger disruption via a number of different mechanisms. The addition of acid may cause degradation or disassembly of the shell wall through a variety of mechanisms. Addition of protons may disassemble cross-linking of polymers in the shell wall, disrupt ionic or hydrogen bonds in the shell wall, or create nanopores in the shell wall to allow the inner contents to leak through to the exterior. In some examples, the microcapsule comprises acid-degradable chemical cross-linkers such a ketals. A decrease in pH, particular to a pH lower than 5, may induce the ketal to convert to a ketone and two alcohols and facilitate disruption of the microcapsule. In other examples, the microcapsules may comprise one or more polyelectrolytes (e.g., PAA, PAAm, PSS, etc.) that are pH sensitive. A decrease in pH may disrupt the ionic- or hydrogen-bonding interactions of such microcapsules, or create nanopores therein. In some cases, microcapsules comprising polyelectrolytes comprise a charged, gel-based core that expands and contracts upon a change of pH.

Removal of cross-linkers (e.g., disulfide bonds) within the microcapsules can also be accomplished through a number of mechanisms. In some examples, various chemicals can be added to a solution of microcapsules that induce either oxidation, reduction or other chemical changes to polymer components of the shell wall. In some cases, a reducing agent, such as beta-mercaptoethanol, dithiotheritol (DTT), or 2-tris(2-carboxyethyl) phosphine (TCEP), is added such that disulfide bonds in a microcapsule shell wall are disrupted. In addition, enzymes may be added to cleave peptide bonds within the microcapsules, thereby resulting in cleavage of shell wall cross linkers.

Depolymerization can also be used to disrupt the microcapsules. A chemical trigger may be added to facilitate the removal of a protecting head group. For example, the trigger may cause removal of a head group of a carbonate ester or carbamate within a polymer, which in turn causes depolymerization and release of reagents from the inside of the capsule.

Shell wall switching reactions may be due to any structural change to the porosity of the shell wall. The porosity of a shell wall may be modified, for example, by the addition of azo dyes or viologen derivatives. Addition of energy (e.g., electricity, light) may also be used to stimulate a change in porosity.

In yet another example, a chemical trigger may comprise an osmotic trigger, whereby a change in ion or solute concentration of microcapsule solution induces swelling of the capsule. Swelling may cause a buildup of internal pressure such that the capsule ruptures to release its contents.

It is also known in the art that bulk or physical changes to the microcapsule through various stimuli also offer many advantages in designing capsules to release reagents. Bulk or physical changes occur on a macroscopic scale, in which capsule rupture is the result of mechano-physical forces induced by a stimulus. These processes may include, but are not limited to pressure induced rupture, shell wall melting, or changes in the porosity of the shell wall.

B. Biological Stimuli

Biological stimuli may also be used to trigger disruption or degradation of microcapsules. Generally, biological triggers resemble chemical triggers, but many examples use biomolecules, or molecules commonly found in living systems such as enzymes, peptides, saccharides, fatty acids, nucleic acids and the like. For example, microcapsules may comprise polymers with peptide cross-links that are sensitive to cleavage by specific proteases. More specifically, one example may comprise a microcapsule comprising GFLGK peptide cross links. Upon addition of a biological trigger such as the protease Cathepsin B, the peptide cross links of the shell well are cleaved and the contents of the capsule are released. In other cases, the proteases may be heat-activated. In another example, microcapsules comprise a shell wall comprising cellulose. Addition of the hydrolytic enzyme

chitosan serves as biologic trigger for cleavage of cellulosic bonds, depolymerization of the shell wall, and release of its inner contents.

C. Thermal Stimuli

The microcapsules may also be induced to release their contents upon the application of a thermal stimulus. A change in temperature can cause a variety changes to the microcapsule. A change in heat may cause melting of a microcapsule such that the shell wall disintegrates. In other cases, the heat may increase the internal pressure of the inner components of the capsule such that the capsule ruptures or explodes. In still other cases, the heat may transform the capsule into a shrunken dehydrated state. The heat may also act upon heat-sensitive polymers within the shell of a microcapsule to cause disruption of the microcapsule.

In one example, a microcapsule comprises a thermosensitive hydrogel shell encapsulating one or more emulsified reagent particles. Upon the application of heat, such as above 35 C, the hydrogel material of the outer shell wall shrinks. The sudden shrinkage of the shell ruptures the capsule and allows the reagents of the inside of the capsule to squirt out in the sample preparation solution in the microwell.

In some cases, the shell wall may comprise a diblock polymer, or a mixture of two polymers, with different heat sensitivities. One polymer may be particularly likely to shrink after the application of heat, while the other is more heat-stable. When heat is applied to such shell wall, the heat-sensitive polymer may shrink, while the other remains intact, causing a pore to form. In still other cases, a shell wall may comprise magnetic nanoparticles. Exposure to a magnetic field may cause the generation of heat, leading to rupture of the microcapsule.

D. Magnetic Stimuli

Inclusion of magnetic nanoparticles to the shell wall of microcapsules may allow triggered rupture of the capsules as well as guide the particles in an array. A device of this disclosure may comprise magnetic particles for either purpose. In one example, incorporation of Fe3O4 nanoparticles into polyelectrolyte containing capsules triggers rupture in the presence of an oscillating magnetic field stimulus.

E. Electrical and Light Stimuli

A microcapsule may also be disrupted or degraded as the result of electrical stimulation. Similar to magnetic particles described in the previous section, electrically sensitive particles can allow for both triggered rupture of the capsules as well as other functions such as alignment in an electric field, electrical conductivity or redox reactions. In one example, microcapsules containing electrically sensitive material are aligned in an electric field such that release of inner reagents can be controlled. In other examples, electrical fields may induce redox reactions within the shell wall itself that may increase porosity.

A light stimulus may also be used to disrupt the microcapsules. Numerous light triggers are possible and may include systems that use various molecules such as nanoparticles and chromophores capable of absorbing photons of specific ranges of wavelengths. For example, metal oxide coatings can be used as capsule triggers. UV irradiation of polyelectrolyte capsules coated with SiO2/TiO2 may result in disintegration of the capsule wall. In yet another example, photo switchable materials such as azobenzene groups may be incorporated in the shell wall. Upon the application of UV or visible light, chemicals such as these undergo a reversible cis-to-trans isomerization upon absorption of photons. In this aspect, incorporation of photo switches result in a shell wall that may disintegrate or become more porous upon the application of a light trigger.

F. Application of Stimuli

A device of this disclosure may be used in combination with any apparatus or device that provides such trigger or stimulus. For example, if the stimulus is thermal, a device may be used in combination with a heated or thermally controlled plate, which allows heating of the microwells and may induce the rupture of capsules. Any of a number of heat transfers may be used for thermal stimuli, including but not limited to applying heat by radiative heat transfer, convective heat transfer, or conductive heat transfer. In other cases, if the stimulus is a biological enzyme, the enzyme may be injected into a device such that it is deposited into each microwell. In another aspect, if the stimulus is a magnetic or electric field, a device may be used in combination with a magnetic or electric plate.

A chemical stimulus may be added to a partition and may exert its function at various times after contacting a chemical stimulus with a microcapsule. The speed at which a chemical stimulus exerts its effect may vary depending on, for example, the amount/concentration of a chemical stimulus contacted with a microcapsule and/or the particular chemical stimulus used. For example, a droplet may comprise a degradable gel bead (e.g., a gel bead comprising chemical cross-linkers, such as, for example, disulfide bonds). Upon droplet formation, a chemical stimulus (e.g., a reducing agent) may be included in the droplet with the gel bead. The chemical stimulus may degrade the gel bead immediately on contact with the gel bead, soon after (e.g., about 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 min) contact with the gel bead, or at a later time. In some cases, degradation of the gel bead may occur before, during, or after a further processing step, such as, for example, a thermal cycling step as described herein.

V. Sample Preparation, Reaction and Recovery

After application of the stimulus, rupturing of capsules and release of the reagents, the sample preparation reaction may proceed in a device. Reactions within a device may be incubated for various periods of times depending on the reagents used in the sample reactions. A device may also be used in combination with other devices that aid in the sample preparation reaction. For example, if PCR amplification is desired, a device may be used in combination with a PCR thermocycler. In some cases, a thermocycler may comprise a plurality of wells. In cases where partitions are droplets, the droplets may be entered into the wells of the thermocycler. In some cases, each well may comprise multiple droplets, such that when thermal cycling is initiated, multiple droplets are thermal cycled in each well. In another example, if the reaction requires agitation, a device may be used in combination with a shaking apparatus.

Following the completion of the sample preparation reaction, the analytes and products of the sample reactions may be recovered. In some cases, a device may utilize a method comprising the application of liquid or gas to flush out the contents of the individual microwells. In one example, the liquid comprises an immiscible carrier fluid that preferentially wets the microwell array material. It may also be immiscible with water so as to flush the reaction products out of the well. In another example, the liquid may be an aqueous fluid that can be used to flush out the samples out of the wells. After flushing of the contents of the microwells, the contents of the microwells are pooled for a variety of downstream analyses and applications.

VI. Applications

FIG. **4**A provides a general flow of many of the methods of the present disclosure; and FIG. **4**B provides a generally annotated version of **4**A. One or more microcapsule(s) that contain reagents **410** may be pre-loaded into microwells, followed by addition of an analyte, which, in this particular Figure, is a nucleic acid analyte **420**. The microwells may then be sealed **430** by any method, such as by application of a sealing fluid. The inlet and outlet ports may also be sealed, for example to prevent evaporation. Following these steps, a stimulus (e.g., heat, chemical, biological, etc.) may be applied to the microwells in order to disrupt the microcapsules **460** and trigger release of the reagents **450** to the interior of the microwell. Subsequently, an incubation step **440** may occur in order to enable the reagents perform a particular function such as lysis of cells, digestion of protein, fragmentation of high molecular weight nucleic acids, or ligation of oligonucleotide bar codes. Following the incubation step (which is optional), the contents of the microwells may be recovered either singly or in bulk.

A. Analytes

A device of this disclosure may have a wide variety of uses in the manipulation, preparation, identification and/or quantification of analytes. In some cases, the analyte is a cell or population of cells. The population of cells may be homogeneous (e.g., from a cell line, of the same cell type, from the same type of tissue, from the same organ, etc.) or heterogenous (mixture of different types of cells). The cells may be primary cells, cell lines, recombinant cells, primary cells, encapsulated cells, free cells, etc.

The analytes may also be molecules, including but not limited to: polypeptides, proteins, antibodies, enzymes, nucleic acids, saccharides, small molecules, drugs, and the like. Examples of nucleic acids include but are not limited to: DNA, RNA, dNTPs, ddNTPs, amplicons, synthetic nucleotides, synthetic polynucleotides, polynucleotides, oligonucleotides, peptide nucleic acids, cDNA, dsDNA, ssDNA, plasmid DNA, cosmid DNA, high Molecular Weight (MW) DNA, chromosomal DNA, genomic DNA, viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), mRNA, rRNA, tRNA, nRNA, siRNA, snRNA, snoRNA, scaRNA, microRNA, dsRNA, ribozyme, riboswitch and viral RNA (e.g., retroviral RNA).

In some cases, the analytes are pre-mixed with one or more additional materials, such as one or more reagents (e.g., ligase, protease, polymerase) prior to being loaded into the device. In some cases, the analytes are pre-mixed with microcapsules comprising one or more reagents prior to being loaded onto the device.

The samples may be derived from a variety of sources including human, mammal, non-human mammal, ape, monkey, chimpanzee, plant, reptilian, amphibian, avian, fungal, viral or bacterial sources. Samples such as cells, nucleic acids and proteins may also be obtained from a variety of clinical sources such as biopsies, aspirates, blood draws, urine samples, formalin fixed embedded tissues and the like.

A device of this disclosure may also enable the analytes to be tagged or tracked in order to permit subsequent identification of an origin of the analytes. This feature is in contrast with other methods that use pooled or multiplex reactions and that only provide measurements or analyses as an average of multiple samples. Here, the physical partitioning and assignment of a unique identifier to individual analytes allows acquisition of data from individual samples and is not limited to averages of samples.

In some examples, nucleic acids or other molecules derived from a single cell may share a common tag or identifier and therefore may be later identified as being derived from that cell. Similarly, all of the fragments from a single strand of nucleic acid may be tagged with the same identifier or tag, thereby permitting subsequent identification of fragments with similar phasing or linkage on the same strand. In other cases, gene expression products (e.g., mRNA, protein) from an individual cell may be tagged in order to quantify expression. In still other cases, the device can be used as a PCR amplification control. In such cases, multiple amplification products from a PCR reaction can be tagged with the same tag or identifier. If the products are later sequenced and demonstrate sequence differences, differences among products with the same identifier can then be attributed to PCR error.

The analytes may be loaded onto the device before, after, or during loading of the microcapsules and/or free reagents. In some cases, the analytes are encapsulated into microcapsules before loading into the microcapsule array. For example, nucleic acid analytes may be encapsulated into a microcapsule, which is then loaded onto the device and later triggered to release the analytes into an appropriate microwell.

Any analytes, such as DNA or cells, may be loaded in solution or as analytes encapsulated in a capsule. In some cases, homogeneous or heterogeneous populations of molecules (e.g., nucleic acids, proteins, etc.) are encapsulated into microcapsules and loaded onto the device. In some cases, homogeneous or heterogeneous populations of cells are encapsulated into microcapsules and loaded onto the device. The microcapsules may comprise a random or specified number of cells and/or molecules. For example, the microcapsules may comprise no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500, 1000, 5000, or 10000 cells and/or molecules per microcapsule. In other examples, the microcapsules comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500, 1000, 5000, or 10000 cells and/or molecules per microcapsule. Fluidic techniques and any other techniques may be used to encapsulate the cells and/or molecules into the microcapsules.

Generally, the methods and compositions provided herein are useful for preparation of an analyte prior to a downstream application such as a sequencing reaction. Often, a sequencing method is classic Sanger sequencing. Sequencing methods may include, but are not limited to: high-throughput sequencing, pyrosequencing, sequencing-by-synthesis, single-molecule sequencing, nanopore sequencing, sequencing-by-ligation, sequencing-by-hybridization, RNA-Seq (Illumina), Digital Gene Expression (Helicos), Next generation sequencing, Single Molecule Sequencing by Synthesis (SMSS)(Helicos), massively-parallel sequencing, Clonal Single Molecule Array (Solexa), shotgun sequencing, Maxim-Gilbert sequencing, primer walking, and any other sequencing methods known in the art.

There are numerous examples of applications that may be conducted instead of, or in conjunction with, a sequencing reaction, including but not limited to: biochemical analyses, proteomics, immunoassays, profiling/fingerprinting of specific cell types, pharmaceutical screening, bait-capture experiments, protein-protein interaction screens and the like.

B. Assignment of Unique Identifiers to Analytes

The devices disclosed herein may be used in applications that involve the assignment of unique identifiers, or molecular bar codes, to analytes. Often, the unique identifier is a bar-code oligonucleotide that is used to tag the analytes; but, in some cases, different unique identifiers are used. For

example, in some cases, the unique identifier is an antibody, in which case the attachment may comprise a binding reaction between the antibody and the analyte (e.g., antibody and cell, antibody and protein, antibody and nucleic acid). In other cases, the unique identifier is a dye, in which case the attachment may comprise intercalation of the dye into the analyte molecule (such as intercalation into DNA or RNA) or binding to a probe labeled with the dye. In still other cases, the unique identifier may be a nucleic acid probe, in which case the attachment to the analyte may comprise a hybridization reaction between the nucleic acid and the analyte. In some cases, the reaction may comprise a chemical linkage between the identifier and the analyte. In other cases, the reaction may comprise addition of a metal isotope, either directly to the analyte or by a probe labeled with the isotope.

Often, the method comprises attaching oligonucleotide bar codes to nucleic acid analytes through an enzymatic reaction such as a ligation reaction. For example, the ligase enzyme may covalently attach a DNA bar code to fragmented DNA (e.g., high molecular-weight DNA). Following the attachment of the bar-codes, the molecules may be subjected to a sequencing reaction.

However, other reactions may be used as well. For example, oligonucleotide primers containing bar code sequences may be used in amplification reactions (e.g., PCR, qPCR, reverse-transcriptase PCR, digital PCR, etc.) of the DNA template analytes, thereby producing tagged analytes. After assignment of bar codes to individual analytes, the contents of individual microwells may be recovered via the outlet port in the device for further analyses.

The unique identifiers (e.g., oligonucleotide bar-codes, antibodies, probes, etc.) may be introduced to the device randomly or nonrandomly. In some cases, they are introduced at an expected ratio of unique identifiers to microwells. For example, the unique identifiers may be loaded so that more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 unique identifiers are loaded per microwell. In some cases, the unique identifiers may be loaded so that less than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 unique identifiers are loaded per microwell. In some cases, the average number of unique identifiers loaded per microwell is less than, or greater than, about 0.0001, 0.001, 0.01, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 unique identifiers per microwell.

The unique identifiers also may be loaded so that a set of one or more identical identifiers are introduced to a particular well. Such sets may also be loaded so that each microwell contains a different set of identifiers. For example, a population of microcapsules may be prepared such that a first microcapsule in the population comprises multiple copies of identical unique identifiers (e.g., nucleic acid bar codes, etc.) and a second microcapsule in the population comprises multiple copies of a unique identifier that differs from within the first microcapsule. In some cases, the population of microcapsules may comprise multiple microcapsules (e.g., greater than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules), each containing multiple copies of a unique identifier that differs from that contained in the other microcapsules. In some cases, the population may comprise greater than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules with identical sets of unique identifiers. In some cases, the population may comprise greater than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules, wherein the microcapsules each comprise a different combination of unique identifiers. For example, in some cases the different combinations overlap, such that a first microcapsule may comprise, e.g., unique identifiers A, B, and C, while a second microcapsule may comprise unique identifiers A, B, and D. In another example, the different combinations do not overlap, such that a first microcapsule may comprise, e.g., unique identifiers A, B, and C, while a second microcapsule may comprise unique identifiers D, E, and F.

The unique identifiers may be loaded into the device at an expected or predicted ratio of unique identifiers per analyte (e.g., strand of nucleic acid, fragment of nucleic acid, protein, cell, etc.) In some cases, the unique identifiers are loaded in the microwells so that more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 unique identifiers are loaded per individual analyte in the microwell. In some cases, the unique identifiers are loaded in the microwells so that less than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 unique identifiers are loaded per individual analyte in the microwell. In some cases, the average number of unique identifiers loaded per analyte is less than, or greater than, about 0.0001, 0.001, 0.01, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 unique identifiers per analyte. When more than one identifier is present per analyte, such identifiers may be copies of the same identifier, or multiple different identifiers. For example, the attachment process may be designed to attach multiple identical identifiers to a single analyte, or multiple different identifiers to the analyte.

The unique identifiers may be used to tag a wide range of analytes, including cells or molecules. For example, unique identifiers (e.g., bar code oligonucleotides) may be attached to whole strands of nucleic acids or to fragments of nucleic acids (e.g., fragmented genomic DNA, fragmented RNA). The unique identifiers (e.g., antibodies, oligonucleotides) may also bind to cells, include the external surface of a cell, a marker expressed on the cell or components within the cell such as organelles, gene expression products, genomic DNA, mitochondrial DNA, RNA, mRNA, or proteins. The unique identifiers also may be designed to bind or hybridize nucleic acids (e.g., DNA, RNA) present in permeabilized cells, which may or may not be otherwise intact.

The unique identifiers may be loaded onto the device either singly or in combination with other elements (e.g., reagents, analytes). In some cases, free unique identifiers are pooled with the analytes and the mixture is loaded into the device. In some cases, unique identifiers encapsulated in microcapsules are pooled with the analytes, prior to loading of the mixture onto the device. In still other cases, free unique identifiers are loaded into the microwells prior to, during (e.g., by separate inlet port), or following the loading of the analytes. In still other cases, unique identifiers encapsulated in microcapsules are loaded into the microwells prior to, concurrently with (e.g., by separate inlet port), or after loading of the analytes.

In many applications, it may be important to determine whether individual analytes each receive a different unique identifier (e.g., oligonucleotide bar code). If the population of unique identifiers introduced into the device is not significantly diverse, different analytes may possibly be tagged with identical identifiers. The devices disclosed herein may enable detection of analytes tagged with the same identifier. In some cases, a reference analyte may be included with the

population of analytes introduced into the device. The reference analyte may be, for example, a nucleic acid with a known sequence and a known quantity. After the population of analytes is loaded and partitioned in the device, unique identifiers may be attached to the analytes, as described herein. If the unique identifiers are oligonucleotide bar codes and the analytes are nucleic acids, the tagged analytes may subsequently be sequenced and quantified. These methods may indicate if one or more fragments and/or analytes may have been assigned an identical bar code.

A method disclosed herein may comprise loading the device with the reagents necessary for the assignment of bar codes to the analytes. In the case of ligation reactions, reagents including, but not limited to, ligase enzyme, buffer, adapter oligonucleotides, a plurality of unique identifier DNA bar codes and the like may be loaded into the device. In the case of enrichment, reagents including but not limited to a plurality of PCR primers, oligonucleotides containing unique identifying sequence, or bar code sequence, DNA polymerase, DNTPs, and buffer and the like may be loaded into the device. The reagents may be loaded as free reagents or as reagents encapsulated in microcapsules.

C. Nucleic Acid Sequencing

Nucleic acid sequencing may begin with the physical partitioning of sample analytes into microwells at a particular density (e.g., about 1 analyte per microwell or other density described herein). When nucleic acid bar codes are assigned to individual analytes, it may then be possible to track individual molecules during subsequent steps such as subsequent amplification and/or sequencing steps, even if the analytes are later pooled together and treated en masse.

a. Nucleic Acid Phasing

The devices provided herein may be used to prepare analytes (e.g., nucleic acid analytes) in such a manner that enables phasing or linkage information to be subsequently obtained. Such information may allow for the detection of linked genetic variations in sequences, including genetic variations (e.g., SNPs, mutations, indels, copy number variations, transversions, translocations, inversions, etc.) that are separated by long stretches of nucleic acids. These variations may exist in either a cis or trans relationship. In cis relationships, two or more genetic variations may exist in the same polynucleic acid molecule or strand. In trans relationships, two or more genetic variations may exist on multiple nucleic acid molecules or strands.

A method of determining nucleic acid phasing may comprise loading a nucleic acid sample (e.g., a nucleic acid sample that spans a given locus or loci) into a device disclosed herein, distributing the sample such that at most one molecule of nucleic acid is present per microwell, and fragmenting the sample within the microwells. The method may further comprise attaching unique identifiers (e.g., bar codes) to the fragmented nucleic acids as described herein, recovering the nucleic acids in bulk, and performing a subsequent sequencing reaction on the samples in order to detect genetic variations, such as two different genetic variations. The detection of genetic variations tagged with two different bar codes may indicate that the two genetic variations are derived from two separate strands of DNA, reflecting a trans relationship. Conversely, the detection of two different genetic variations tagged with the same bar codes may indicate that the two genetic variations are from the same strand of DNA, reflecting a cis relationship.

Phase information may be important for the characterization of the analyte, particularly if the analyte derives from a subject at risk of, having, or suspected of a having a particular disease or disorder (e.g., hereditary recessive disease such as Cystic Fibrosis, cancer, etc.). The information may be able to distinguish between the following possibilities: (1) two genetic variations within the same gene on the same strand of DNA and (2) two genetic variations within the same gene but located on separate strands of DNA. Possibility (1) may indicate that one copy of the gene is normal and the individual is free of the disease, while possibility (2) may indicate that the individual has or will develop the disease, particularly if the two genetic variations are damaging to the function of the gene when present within the same gene copy. Similarly, the phasing information may also be able to distinguish between the following possibilities: (1) two genetic variations, each within a different gene on the same strand of DNA and (2) two genetic variations, each within a different gene but located on separate strands of DNA.

b. Cell-Specific Information

The devices provided herein may be used to prepare cellular analytes in such a manner that enables cell-specific information to be subsequently obtained. Such information may enable detection of genetic variations (e.g., SNPs, mutations, indels, copy number variations, transversions, translocations, inversions, etc.) on a cell-by-cell basis, thereby enabling a determination of whether the genetic variation(s) are present in the same cell or two different cells.

A method of determining nucleic acid cell-specific information may comprise loading a cellular sample (e.g., a cellular sample from a subject) into a device disclosed herein, distributing the sample such that at most one cell is present per microwell, lysing the cells, and then tagging the nucleic acids within the cells with unique identifiers using a method described herein. In some cases, microcapsules comprising unique identifiers are loaded in the microwell array device (either before, during, or after the loading of the cellular analytes) in such a manner that each cell is contacted with a different microcapsule. The resulting tagged nucleic acids can then be pooled, sequenced, and used to trace the origin of the nucleic acids. Nucleic acids with identical unique identifiers may be determined to originate from the same cell, while nucleic acids with different unique identifiers may be determined to originate from different cells.

In a more specific example, the methods herein may be used to detect the distribution of oncogenic mutations across a population of cancer tumor cells. In this example, some of the cells may have a mutation, or amplification, of an oncogene (e.g., HER2, BRAF, EGFR, KRAS) on two strands of DNA (homozygous), while others may be heterozygous for the mutation, while still other cells may be wild-type and comprise no mutations or other variation in the oncogene. The methods described herein may be able to detect these differences, and also may enable quantification of the relative numbers of homozygous, heterozygous, and wild-type cells. Such information may be used to stage a particular cancer or to monitor the progression of the cancer over time.

In some examples, this disclosure provides methods of identifying mutations in two different oncogenes (e.g., KRAS and EGFR). If the same cell comprises genes with both mutations, this may indicate a more aggressive form of cancer. In contrast, if the mutations are located in two different cells, this may indicate that the cancer is more benign, or less advanced.

The following is another specific example of cell-specific sequence determination. In this example, a plurality of cells, such as from a tumor biopsy, is loaded into a device. Single cells from the sample are deposited into individual wells and labeled with a DNA bar code.

Loading of cells into a device may be achieved through non-random loading. Parameters for non-random loading of analytes, such as cells, may be understood using an interference function such that:

$$\text{“fraction multi-occupancy”} = 1 - \left[\left(1 - \frac{1}{N}\right) + \frac{P}{N}\right]^{C}$$

where

P=probability that a particular cell will attempt but not fit in the well (measure of interference)

N=number of wells

L=number of labels=barcodes

C=number of cells

As part of sample preparation reactions, cells may be lysed and many subsequent reactions are possible, including RNA amplification, DNA amplification or antibody screening for different target proteins and genes in individual cells. After the reaction, the contents of the cells may be pooled together and could be further analyzed, such as by DNA sequencing. With each cell assigned a unique barcode, further analyses may be possible including but not limited to quantification of different gene levels or nucleic acid sequencing of individual cells. In this example, it may be determined whether the tumor comprises cells with different genetic backgrounds (e.g., cancer clones and subclones). The relative number of each type of cell may also be calculated.

c. Amplification Control

As disclosed herein, the device can be used for purposes of controlling for amplification errors, such as PCR errors. For example, a nucleic acid sample may be partitioned into the microwells of the device. Following partitioning, the sample may be subjected to a PCR amplification reaction within the microwells. The PCR products within a microwell may be tagged with the same unique identifier, using a method described herein. If the products are later sequenced and demonstrate sequence differences, differences among products with the same identifier can then be attributed to PCR error.

d. Gene-Expression Products Analysis

In other applications, a device may be used to detect gene product (e.g., protein, mRNA) expression levels in a sample, often on a cell-by-cell basis. A sample may comprise individual cells, a pool of mRNA extract from cells, or other collection of gene products. In some instances, single cells may be loaded into microwells. In other instances, a pool of mRNA or other gene product may be loaded such that a desired quantity of mRNA molecules is loaded into individual microwells.

The methods provided herein may be particularly useful for RNA analysis. For example, using the methods provided herein, unique identifiers may be assigned to mRNA analytes either directly or to cDNA products of a reverse transcription reaction performed on the mRNA analytes. The reverse transcription reaction may be conducted within the microwells of the device following loading of the analytes. Reagents for the reaction may include but are not limited to reverse transcriptase, DNA polymerase enzyme, buffer, dNTPs, oligonucleotide primers, oligonucleotide primers containing bar code sequences and the like. One or more reagents may be loaded into microcapsules or loaded freely in solution into the device or a combination thereof. Sample preparation may then be conducted, such as by fragmenting the cDNA and attaching unique identifiers to the fragments. After sample preparation and recovery, the nucleic acid products of the reaction may be further analyzed, such as by sequencing.

Additionally, a device may be used to characterize multiple cell markers, similar to a flow cytometer. Any cell marker may be characterized, including cell-surface markers (e.g., extracellular proteins, transmembrane markers) and markers located within the internal portion of a cell (e.g., RNA, mRNA, microRNA, multiple copies of genes, proteins, alternative splicing products, etc.). For example, cells may be partitioned within the device, as described herein, so that at most one cell is present within a microwell. Cell markers such as nucleic acids (e.g., RNA) may be extracted and/or fragmented prior to being labeled with a unique identifier (e.g., molecular bar code). Or, alternatively, the nucleic acids may be labeled with a unique identifier without being extracted and/or fragmented. The nucleic acids may then be subjected to further analysis such as sequencing reactions designed to detect multiple gene expression products. Such analysis may be useful in a number of fields. For example, if the starting cells are immune cells (e.g., T cells, B cells, macrophages, etc.), the analysis may provide information regarding multiple expressed markers and enable immunophenotyping of the cells, for example by identifying different CD markers of the cells (e.g., CD3, CD4, CD8, CD19, CD20, CD 56, etc.). Such markers can provide insights into the function, character, class, or relative maturity of the cell. Such markers can also be used in conjunction with markers that are not necessarily immunophenotyping markers, such as markers of pathogenic infection (e.g., viral or bacterial protein, DNA, or RNA). In some cases, the device may be used to identify at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 500, 700, 1000, 5000, 10000, 50000, or 100000 different gene expression products or other form of cellular markers on a single-cell basis. Often, such methods do not comprise use of dyes or probes (e.g., fluorescent probes or dyes).

Gene expression product analysis may be useful in numerous fields including immunology, cancer biology (e.g., to characterize the existence, type, stage, aggressiveness, or other characteristic of cancerous tissue), stem cell biology (e.g., in order to characterize the differentiation state of a stem cell, potency of a stem cell, cellular type of a stem cell, or other features of a stem cell), microbiology, and others. The gene expression analysis may also be used in drug screening applications, for example to evaluate the effect of a particular drug or agent on the gene expression profile of particular cells.

VII. Terminology

The terminology used therein is for the purpose of describing particular embodiments only and is not intended to be limiting of a device of this disclosure. As used herein, the singular forms “a”, “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms “including”, “includes”, “having”, “has”, “with”, or variants thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner similar to the term “comprising”.

Several aspects of a device of this disclosure are described above with reference to example applications for illustration. It should be understood that numerous specific details, relationships, and methods are set forth to provide a full understanding of a device. One having ordinary skill in the relevant art, however, will readily recognize that a device

can be practiced without one or more of the specific details or with other methods. This disclosure is not limited by the illustrated ordering of acts or events, as some acts may occur in different orders and/or concurrently with other acts or events. Furthermore, not all illustrated acts or events are required to implement a methodology in accordance with this disclosure.

Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. The term "about" as used herein refers to a range that is 15% plus or minus from a stated numerical value within the context of the particular usage. For example, about 10 would include a range from 8.5 to 11.5.

The term microwell array, as used herein, generally refers to a predetermined spatial arrangement of microwells. Microwell array devices that comprise a microcapsule may also be referred to as "microwell capsule arrays." Further, the term "array" may be used herein to refer to multiple arrays arranged on a surface, such as would be the case where a surface has multiple copies of an array. Such surfaces bearing multiple arrays may also be referred to as "multiple arrays" or "repeating arrays."

EXAMPLE 1

Single Cell DNA Sequencing

A microwell capsule array is prepared to perform nucleic acid sequencing on individual human B-cells taken from a blood sample. Approximately 15,000 cells are harvested and used for loading into the device. A device of this disclosure and containing 150,000 microwells is used. Each well is cylindrical in shape having a diameter of 125 um and a height of 125 um, allowing at most 1 capsule to be loaded per well. Microcapsules made through emulsion polymerization with a PNIPAM hydrogel shell wall are created such that the microcapsules have a diameter of 100 um for loading in the device. The microcapsules are created such that the PNIPAM shell contains magnetic iron particles. The outer surface of the shell is then chemically coupled to a antibody specific to a transmembrane B cell receptor on the outside of a B cell.

During the preparation process of capsules, reagents are simultaneously loaded into the capsules. Reagents necessary for cell lysis and labeling individual DNA strands of the cells with DNA barcodes are loaded into capsules. Reagents for cell lysis include a mild non-ionic detergent, buffer and salt. Reagents for the addition of DNA bar codes to genomic DNA included restriction enzymes, ligase, and >10,000,000 unique DNA oligonucleotides are loaded into capsules. Capsules are designed to be sensitive to rupture at greater than 65 C.

Capsules are prepared to be applied to the microcapsule array. The array is placed on a magnetic temperature controlled hot plate. Microcapsules are added to a sample of B cells such that one B cell is able to bind to one capsule. Capsule-cell conjugates are applied in aqueous carrier solution in a quantity in excess to the relative number of wells. Gentle pipetting of capsules-cells into the inlet port followed by application of a vacuum manifold to the outlet port distributes the capsules throughout the device. A magnetic field is applied through the plate. Excess capsule-cell solution is removed via pipetting through the outlet port. Each capsule-cell conjugate is trapped and positioned in individual wells via the magnetic field.

After the cells and capsules are loaded in the device, a carrier oil (or sealing fluid) is applied to the device to remove any excess aqueous solution bridging adjacent microwells. The carrier oil applied to the inlet and excess oil is recovered at the outlet with a vacuum manifold. After the carrier oil is applied, the inlet and outlet ports are sealed with tape.

The device is then heated, via the magnetic temperature controlled hot plate, to a temperature of 70 C for 10 min to allow for capsule rupture and cell lysis. The hot plate is then switched to 37 C, for restriction and ligation, for up to 1 hour.

After the sample preparation reaction is completed, the contents of the wells are recovered. The inlet and outlet ports of the device are unsealed and nitrogen gas is applied to the device to flush out the individual components of the microwells. The sample is collected in bulk via a pipette at the outlet port, while the magnetic field retains ruptured capsule shells in individual microwells.

The sample is then sequenced using a multiplex sequencing strategy known in the art. Bar coding of individual cells allows for sequencing information to be gained for individual cells rather than as an average of multiple cells. Based upon the number of cells sequenced and bar codes assigned, SNP cell-specific information is gained. Moreover, the number of reads for individual bar codes can be counted to provide insight into the distribution of different types of cells with varying genetic backgrounds, within the original population of B cells.

EXAMPLE 2

DNA Single Strand Sequencing

A microwell capsule array is prepared to perform nucleic acid sequencing on individual strands of DNA isolated from a population of human skin cells. Cells are lysed using detergent and heat and approximately 15,000 copies of diploid DNA are precipitated via chloroform/ethanol extraction. A resuspension of DNA is loaded into the device with approximately 10,000 copies of haploid DNA. A device of this disclosure, with 300,000 microwells is used. Each well is cylindrical in shape having a diameter of 125 um and a height of 125 um, allowing at most 1 capsule to be loaded per well. Microcapsules made through emulsion polymerization with a PNIPAM hydrogel shell wall are created to a specification of a sphere with a diameter of 100 um for loading into the device.

During the preparation of the microcapsules, reagents are simultaneously loaded into the capsules. The reagents include reagents necessary for labeling individual DNA strands with DNA barcodes, including restriction enzymes, ligase, and >10,000,000 unique DNA oligonucleotides. Capsules designed to be sensitive to rupture at greater than 65 C are used for the encapsulation.

Capsules are applied aqueous carrier solution in an excess to the relative number of wells. Gentle pipetting of capsules into the inlet followed by application of a vacuum manifold to the outlet distributed the capsules throughout the device.

After excess capsule solution is removed, a suspension of DNA in buffer is applied to the device in a similar fashion as the capsules.

After the DNA strands and capsules are loaded in the device, a carrier oil is applied to the device to remove any excess aqueous solution bridging adjacent microwells. The carrier oil is applied to the inlet port and excess oil is recovered at the outlet port with a vacuum manifold. After the carrier oil is applied, the inlet and outlet ports are sealed with tape.

The device is then placed on a temperature controlled hot plate and heated to temperature of 70 C for 10 min to allow for capsule rupture. Reagents are released into the sample preparation reaction. The hot plate is then switched to 37 C, for restriction and ligation, for up to 1 hour.

After the sample preparation reaction is completed, the inlet and outlet ports of the device are unsealed and nitrogen gas is applied to the device to flush out the individual components of the microwells. The sample products, en bulk, are collected via pipette at the outlet port.

The sample is then sequenced to sufficient coverage (e.g., 500) using a multiplex sequencing strategy known in the art. Bar coding of individual DNA strands allows for sequencing information to be gained from individual strands rather than as an average of entire sample of DNA. Based upon the number of DNA strands sequenced and bar codes assigned, SNP phasing/haplotyping information is gained and many repetitive regions of DNA can be resolved. In addition, a substantial boost in accuracy can be gained by discarding mutations that appear randomly with respect to haplotypes, as those are likely to be sequencing errors.

It should be understood from the foregoing that, while particular implementations have been illustrated and described, various modifications may be made thereto and are contemplated herein. It is also not intended that the invention be limited by the specific examples provided within the specification. While the invention has been described with reference to the aforementioned specification, the descriptions and illustrations of the preferable embodiments herein are not meant to be construed in a limiting sense. Furthermore, it shall be understood that all aspects of the invention are not limited to the specific depictions, configurations or relative proportions set forth herein which depend upon a variety of conditions and variables. Various modifications in form and detail of the embodiments of the invention will be apparent to a person skilled in the art. It is therefore contemplated that the invention shall also cover any such modifications, variations and equivalents. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

What is claimed is:

1. A method for sample preparation, comprising:

a) providing a droplet comprising a porous gel bead and a target nucleic acid analyte, wherein said porous gel bead comprises at least 1,000,000 oligonucleotide molecules comprising barcode sequences, wherein said oligonucleotide molecules are releasably attached to said porous gel bead, wherein said barcode sequences are the same sequence for said oligonucleotide molecules;

b) applying a stimulus to said porous gel bead to release said oligonucleotide molecules from said porous gel bead into said droplet, wherein upon release from said porous gel bead, a given oligonucleotide molecule from said oligonucleotide molecules attaches to said target nucleic acid analyte; and

c) subjecting said given oligonucleotide molecule attached to said target nucleic acid analyte to nucleic acid amplification to yield a barcoded target nucleic acid analyte.

2. The method of claim **1**, wherein said droplet is a an aqueous droplet in a continuous oil phase.

3. The method of claim **1**, wherein said oligonucleotide molecules are attached to the porous gel bead via a labile moiety.

4. The method of claim **3**, wherein said labile moiety is a disulfide bond.

5. The method of claim **1**, wherein said stimulus is selected from the group consisting of a biological stimulus, a chemical stimulus, a thermal stimulus, an electrical stimulus, a magnetic stimulus, and a photo stimulus.

6. The method of claim **5**, wherein said stimulus is a chemical stimulus that is a reducing agent.

7. The method of claim **6**, wherein said reducing agent is dithiothreitol (DTT) or tris(2-carboxyethyl) phosphine (TCEP).

8. The method of claim **1**, wherein said given oligonucleotide molecule of said oligonucleotide molecules comprises a region which functions as a primer during said nucleic acid amplification in c).

9. The method of claim **8**, wherein said region which functions as said primer has a sequence for random priming.

10. The method of claim **8**, wherein said primer is configured to amplify said target nucleic acid analyte, thereby producing said barcoded target nucleic acid analyte.

11. The method of claim **1**, wherein said droplet further comprises a polymerase.

12. The method of claim **11**, wherein said oligonucleotide molecules comprise uracil and said polymerase does not recognize uracil.

13. The method of claim **1**, wherein said target nucleic acid analyte is selected from the group consisting of DNA, RNA, amplicons, synthetic polynucleotides, polynucleotides, oligonucleotides, cDNA, dsDNA, ssDNA, plasmid DNA, cosmid DNA, High Molecular Weight (MW) DNA, chromosomal DNA, genomic DNA, viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), mRNA, rRNA, tRNA, nRNA, siRNA, snRNA, snoRNA, scaRNA, microRNA, dsRNA, ribozyme, riboswitch and viral RNA.

14. The method of claim **1**, wherein said oligonucleotide molecules are coupled to said porous gel bead via a covalent bond.

15. The method of claim **1**, wherein said oligonucleotide molecules are reversibly immobilized to said porous gel bead.

16. The method of claim **1**, wherein said droplet in (a) comprises a plurality of target nucleic acid analytes, which plurality of target nucleic acid analytes comprises said target nucleic acid analyte.

17. The method of claim **16**, wherein each of said plurality of target nucleic acid analytes attaches to an individual oligonucleotide molecule of said plurality of oligonucleotide molecules.

18. The method of claim **16**, further comprising fragmenting a nucleic acid sample to yield said plurality of target nucleic acid analytes.

19. The method of claim **1**, wherein said given oligonucleotide molecule from said oligonucleotide molecules attaches to said target nucleic acid analyte by hybridization.

20. The method of claim **1**, further comprising, prior to (a), providing a nucleic acid sample and fragmenting said nucleic acid sample to yield said target nucleic acid analyte.

21. The method of claim **1**, wherein said porous gel bead comprises a polymer gel.

22. The method of claim **21**, wherein said polymer gel is a polyacrylamide.

* * * * *

JX-0005

US009695468B2

(12) **United States Patent**
Hindson et al.

(10) **Patent No.: US 9,695,468 B2**
(45) **Date of Patent: *Jul. 4, 2017**

(54) **METHODS FOR DROPLET-BASED SAMPLE PREPARATION**

(71) Applicant: **10X Genomics, Inc.**, Pleasanton, CA (US)

(72) Inventors: **Benjamin Hindson**, Pleasanton, CA (US); **Serge Saxonov**, Oakland, CA (US); **Michael Schnall-Levin**, San Francisco, CA (US)

(73) Assignee: **10X GENOMICS, INC.**, Pleasanton, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **14/624,473**

(22) Filed: **Feb. 17, 2015**

(65) **Prior Publication Data**

US 2015/0225778 A1 Aug. 13, 2015

Related U.S. Application Data

(63) Continuation of application No. 13/966,150, filed on Aug. 13, 2013.

(60) Provisional application No. 61/683,192, filed on Aug. 14, 2012, provisional application No. 61/737,374, filed on Dec. 14, 2012, provisional application No. 61/762,435, filed on Feb. 8, 2013, provisional application No. 61/800,223, filed on Mar. 15, 2013, provisional application No. 61/840,403, filed on Jun. 27, 2013, provisional application No. 61/844,804, filed on Jul. 10, 2013.

(51) **Int. Cl.**

C12Q 1/68	(2006.01)
C12P 19/34	(2006.01)
C07H 21/04	(2006.01)
G01N 15/06	(2006.01)
C40B 50/14	(2006.01)
C12N 15/10	(2006.01)
B01L 3/00	(2006.01)
B01J 19/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C12Q 1/6806*** (2013.01); ***B01J 19/0046*** (2013.01); ***B01L 3/508*** (2013.01); ***B01L 3/502715*** (2013.01); ***B01L 3/523*** (2013.01); ***C12N 15/1065*** (2013.01); *B01L 2200/0647* (2013.01); *B01L 2400/0677* (2013.01)

(58) **Field of Classification Search**

CPC ... C12C 1/68; B01L 3/05; B01J 13/00; G01N 33/586

USPC 435/6.1, 91.1, 91.2, 287.2, 288.5; 536/24.3, 24.33; 422/68.1; 506/16, 30, 506/31

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,582,802	A	4/1986	Zimmerman et al.
5,149,625	A	9/1992	Church et al.
5,202,231	A	4/1993	Drmanac et al.
5,413,924	A	5/1995	Kosak et al.
5,436,130	A	7/1995	Mathies et al.
5,512,131	A	4/1996	Kumar et al.
5,587,128	A	12/1996	Wilding et al.
5,605,793	A	2/1997	Stemmer
5,618,711	A	4/1997	Gelfand et al.
5,695,940	A	12/1997	Drmanac et al.
5,736,330	A	4/1998	Fulton
5,756,334	A	5/1998	Perler et al.
5,834,197	A	11/1998	Parton
5,851,769	A	12/1998	Gray et al.
5,856,174	A	1/1999	Lipshutz et al.
5,958,703	A	9/1999	Dower et al.
5,994,056	A	11/1999	Higuchi et al.
6,046,003	A	4/2000	Mandecki
6,051,377	A	4/2000	Mandecki
6,057,107	A	5/2000	Fulton
6,103,537	A	8/2000	Ullman et al.
6,143,496	A	11/2000	Brown et al.
6,172,218	B1	1/2001	Brenner
6,265,552	B1	7/2001	Schatz
6,297,006	B1	10/2001	Drmanac et al.
6,297,017	B1	10/2001	Schmidt et al.
6,327,410	B1	12/2001	Walt et al.
6,355,198	B1	3/2002	Kim et al.
6,361,950	B1	3/2002	Mandecki
6,372,813	B1	4/2002	Johnson et al.
6,406,848	B1	6/2002	Bridgham et al.
6,432,360	B1	8/2002	Church
6,485,944	B1	11/2002	Church et al.
6,511,803	B1	1/2003	Church et al.

(Continued)

FOREIGN PATENT DOCUMENTS

EP	0249007 A2	12/1987
EP	0271281 A2	6/1988

(Continued)

OTHER PUBLICATIONS

Aaron P. Esser-Kahn, Triggered Release from Polymer Capsules, 2011, Macromolecules, 44, 5539-5553.*

(Continued)

Primary Examiner — Narayan Bhat

(74) *Attorney, Agent, or Firm* — Wilson Sonsini Goodrich & Rosati

(57) **ABSTRACT**

This disclosure provides microwell capsule array devices. The microwell capsule array devices are generally capable of performing one or more sample preparation operations. Such sample preparation operations may be used as a prelude to one more or more analysis operations. For example, a device of this disclosure can achieve physical partitioning and discrete mixing of samples with unique molecular identifiers within a single unit in preparation for various analysis operations. The device may be useful in a variety of applications and most notably nucleic-acid-based sequencing, detection and quantification of gene expression and single-cell analysis.

23 Claims, 4 Drawing Sheets

10XG-0000054853

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,524,456 B1 2/2003 Ramsey et al.
6,586,176 B1 7/2003 Trnovsky et al.
6,632,606 B1 10/2003 Ullman et al.
6,632,655 B1 10/2003 Mehta et al.
6,670,133 B2 12/2003 Knapp et al.
6,767,731 B2 7/2004 Hannah
6,800,298 B1 10/2004 Burdick et al.
6,806,052 B2 10/2004 Bridgham et al.
6,806,058 B2 10/2004 Jesperson et al.
6,859,570 B2 2/2005 Walt et al.
6,884,788 B2 4/2005 Bulpitt et al.
6,913,935 B1 7/2005 Thomas
6,929,859 B2 8/2005 Chandler et al.
6,969,488 B2 11/2005 Bridgham et al.
6,974,669 B2 12/2005 Mirkin et al.
7,041,481 B2 5/2006 Anderson et al.
7,115,400 B1 10/2006 Adessi et al.
7,129,091 B2 10/2006 Ismagilov et al.
7,268,167 B2 9/2007 Higuchi et al.
7,282,370 B2 10/2007 Bridgham et al.
7,297,485 B2 11/2007 Bornarth et al.
7,323,305 B2 1/2008 Leamon et al.
7,425,431 B2 9/2008 Church et al.
7,536,928 B2 5/2009 Kazuno
7,604,938 B2 10/2009 Takahashi et al.
7,622,280 B2 11/2009 Holliger et al.
7,638,276 B2 12/2009 Griffiths et al.
7,645,596 B2 1/2010 Williams et al.
7,666,664 B2 2/2010 Sarofim et al.
7,708,949 B2 5/2010 Stone et al.
7,709,197 B2 5/2010 Drmanac
7,745,178 B2 6/2010 Dong et al.
7,776,927 B2 8/2010 Chu et al.
RE41,780 E 9/2010 Anderson et al.
7,799,553 B2 9/2010 Mathies et al.
7,842,457 B2 11/2010 Berka et al.
7,901,891 B2 3/2011 Drmanac et al.
7,910,354 B2 3/2011 Drmanac et al.
7,960,104 B2 6/2011 Drmanac et al.
7,968,287 B2 6/2011 Griffiths et al.
7,972,778 B2 7/2011 Brown et al.
8,003,312 B2 8/2011 Krutzik et al.
8,053,192 B2 11/2011 Bignell et al.
8,067,159 B2 11/2011 Brown et al.
8,133,719 B2 3/2012 Drmanac et al.
8,252,539 B2 8/2012 Quake et al.
8,268,564 B2 9/2012 Roth et al.
8,273,573 B2 9/2012 Ismagilov et al.
8,278,071 B2 10/2012 Brown et al.
8,304,193 B2 11/2012 Ismagilov et al.
8,329,407 B2 12/2012 Ismagilov et al.
8,337,778 B2 12/2012 Stone et al.
8,592,150 B2 11/2013 Drmanac et al.
8,603,749 B2 12/2013 Gillevet
8,679,756 B1 3/2014 Brenner et al.
8,748,094 B2 6/2014 Weitz et al.
8,748,102 B2 6/2014 Berka et al.
8,765,380 B2 7/2014 Berka et al.
8,822,148 B2 9/2014 Ismagliov et al.
8,835,358 B2 9/2014 Fodor et al.
8,871,444 B2 10/2014 Griffiths et al.
8,889,083 B2 11/2014 Ismagilov et al.
8,986,286 B2 3/2015 Tanghoj et al.
9,012,390 B2 4/2015 Holtze et al.
9,017,948 B2 4/2015 Agresti et al.
9,029,083 B2 5/2015 Griffiths et al.
9,249,460 B2 2/2016 Pushkarev et al.
9,347,059 B2 5/2016 Saxonov
9,388,465 B2 7/2016 Hindson et al.
9,410,201 B2 8/2016 Hindson et al.
9,567,631 B2 2/2017 Hindson et al.
2001/0020588 A1 9/2001 Adourian et al.
2001/0044109 A1 11/2001 Mandecki
2002/0034737 A1 3/2002 Drmanac
2002/0051992 A1 5/2002 Bridgham et al.
2002/0089100 A1 7/2002 Kawasaki
2002/0092767 A1 7/2002 Bjornson et al.
2002/0179849 A1 12/2002 Maher et al.
2003/0008285 A1 1/2003 Fischer
2003/0008323 A1 1/2003 Ravkin et al.
2003/0027221 A1 2/2003 Scott et al.
2003/0028981 A1 2/2003 Chandler et al.
2003/0039978 A1 2/2003 Hannah
2003/0044777 A1 3/2003 Beattie
2003/0044836 A1 3/2003 Levine et al.
2003/0082587 A1 5/2003 Seul et al.
2003/0104466 A1 6/2003 Knapp et al.
2003/0108897 A1 6/2003 Drmanac
2003/0149307 A1 8/2003 Hai et al.
2003/0170698 A1 9/2003 Gascoyne et al.
2003/0182068 A1 9/2003 Battersby et al.
2003/0207260 A1 11/2003 Trnovsky et al.
2003/0215862 A1 11/2003 Parce et al.
2004/0063138 A1 4/2004 McGinnis et al.
2004/0132122 A1 7/2004 Banerjee et al.
2004/0258701 A1 12/2004 Dominowski et al.
2005/0019839 A1 1/2005 Jespersen et al.
2005/0042625 A1 2/2005 Schmidt et al.
2005/0079510 A1 4/2005 Berka et al.
2005/0130188 A1 6/2005 Walt et al.
2005/0172476 A1 8/2005 Stone et al.
2005/0181379 A1 8/2005 Su et al.
2005/0202429 A1 9/2005 Trau et al.
2005/0202489 A1 9/2005 Cho et al.
2005/0221339 A1 10/2005 Griffiths et al.
2005/0244850 A1 11/2005 Huang et al.
2005/0287572 A1 12/2005 Mathies et al.
2006/0002890 A1 1/2006 Hersel et al.
2006/0020371 A1 1/2006 Ham et al.
2006/0073487 A1 4/2006 Oliver et al.
2006/0078888 A1 4/2006 Griffiths et al.
2006/0153924 A1 7/2006 Griffiths et al.
2006/0163385 A1 7/2006 Link et al.
2006/0199193 A1 9/2006 Koo et al.
2006/0240506 A1 10/2006 Kushmaro et al.
2006/0257893 A1 11/2006 Takahashi et al.
2006/0263888 A1 11/2006 Fritz et al.
2006/0292583 A1 12/2006 Schneider et al.
2007/0003442 A1 1/2007 Link et al.
2007/0020617 A1 1/2007 Trnovsky et al.
2007/0054119 A1 3/2007 Garstecki et al.
2007/0077572 A1 4/2007 Tawfik et al.
2007/0092914 A1 4/2007 Griffiths et al.
2007/0099208 A1 5/2007 Drmanac et al.
2007/0154903 A1 7/2007 Marla et al.
2007/0160503 A1 7/2007 Sethu et al.
2007/0172873 A1 7/2007 Brenner et al.
2007/0190543 A1 8/2007 Livak
2007/0195127 A1 8/2007 Ahn et al.
2007/0207060 A1 9/2007 Zou et al.
2007/0228588 A1 10/2007 Noritomi et al.
2007/0264320 A1 11/2007 Lee et al.
2008/0003142 A1 1/2008 Link et al.
2008/0004436 A1 1/2008 Tawfik et al.
2008/0014589 A1 1/2008 Link et al.
2008/0213766 A1 9/2008 Brown et al.
2008/0228268 A1 9/2008 Shannon et al.
2008/0241820 A1 10/2008 Krutzik et al.
2009/0005252 A1 1/2009 Drmanac et al.
2009/0011943 A1 1/2009 Drmanac et al.
2009/0012187 A1 1/2009 Chu et al.
2009/0025277 A1 1/2009 Takanashi
2009/0035770 A1 2/2009 Mathies et al.
2009/0048124 A1 2/2009 Leamon et al.
2009/0053169 A1 2/2009 Castillo et al.
2009/0068170 A1 3/2009 Weitz et al.
2009/0098555 A1 4/2009 Roth et al.
2009/0118488 A1 5/2009 Drmanac et al.
2009/0137404 A1 5/2009 Drmanac et al.
2009/0137414 A1 5/2009 Drmanac et al.
2009/0143244 A1 6/2009 Bridgham et al.
2009/0148961 A1 6/2009 Luchini et al.
2009/0155781 A1 6/2009 Drmanac et al.
2009/0197248 A1 8/2009 Griffiths et al.

(56) **References Cited**

U.S. PATENT DOCUMENTS

2009/0197772	A1	8/2009	Griffiths et al.
2009/0202984	A1	8/2009	Cantor
2009/0203531	A1	8/2009	Kurn
2009/0264299	A1	10/2009	Drmanac et al.
2009/0286687	A1	11/2009	Dressman et al.
2010/0021973	A1	1/2010	Makarov et al.
2010/0021984	A1	1/2010	Edd et al.
2010/0022414	A1	1/2010	Link et al.
2010/0035254	A1	2/2010	Williams
2010/0062494	A1	3/2010	Church et al.
2010/0069263	A1	3/2010	Shendure et al.
2010/0105112	A1	4/2010	Holtz et al.
2010/0130369	A1	5/2010	Shenderov et al.
2010/0136544	A1	6/2010	Agresti et al.
2010/0137163	A1	6/2010	Link et al.
2010/0173394	A1	7/2010	Colston, Jr. et al.
2010/0210479	A1	8/2010	Griffiths et al.
2011/0033548	A1	2/2011	Lai et al.
2011/0033854	A1	2/2011	Drmanac et al.
2011/0053798	A1	3/2011	Hindson et al.
2011/0071053	A1	3/2011	Drmanac et al.
2011/0086780	A1	4/2011	Colston, Jr. et al.
2011/0092376	A1	4/2011	Colston, Jr. et al.
2011/0092392	A1	4/2011	Colston, Jr. et al.
2011/0160078	A1	6/2011	Fodor et al.
2011/0195496	A1	8/2011	Muraguchi et al.
2011/0201526	A1	8/2011	Berka et al.
2011/0217736	A1	9/2011	Hindson
2011/0218123	A1	9/2011	Weitz et al.
2011/0263457	A1	10/2011	Krutzik et al.
2011/0267457	A1	11/2011	Weitz et al.
2011/0281738	A1	11/2011	Drmanac et al.
2011/0305761	A1	12/2011	Shum et al.
2011/0319281	A1	12/2011	Drmanac
2012/0000777	A1	1/2012	Garrell et al.
2012/0010098	A1	1/2012	Griffiths et al.
2012/0010107	A1	1/2012	Griffiths et al.
2012/0015382	A1	1/2012	Weitz et al.
2012/0015822	A1	1/2012	Weitz et al.
2012/0071331	A1	3/2012	Casbon et al.
2012/0121481	A1	5/2012	Romanowsky et al.
2012/0132288	A1	5/2012	Weitz et al.
2012/0135893	A1	5/2012	Drmanac et al.
2012/0172259	A1	7/2012	Rigatti et al.
2012/0190032	A1	7/2012	Ness et al.
2012/0196288	A1	8/2012	Beer
2012/0211084	A1	8/2012	Weitz et al.
2012/0220494	A1	8/2012	Samuels et al.
2012/0220497	A1	8/2012	Jacobson et al.
2012/0222748	A1	9/2012	Weitz et al.
2012/0309002	A1	12/2012	Link
2012/0316074	A1	12/2012	Saxonov
2013/0018970	A1	1/2013	Woundy et al.
2013/0028812	A1	1/2013	Prieto et al.
2013/0046030	A1	2/2013	Rotem et al.
2013/0078638	A1	3/2013	Berka et al.
2013/0079231	A1	3/2013	Pushkarev et al.
2013/0109575	A1	5/2013	Kleinschmidt et al.
2013/0157870	A1	6/2013	Pushkarev et al.
2013/0157899	A1	6/2013	Adler, Jr. et al.
2013/0178368	A1	7/2013	Griffiths et al.
2013/0189700	A1	7/2013	So et al.
2013/0203675	A1	8/2013	Desimone et al.
2013/0210639	A1	8/2013	Link et al.
2013/0210991	A1	8/2013	Fonnum et al.
2013/0211055	A1	8/2013	Raines et al.
2013/0225418	A1	8/2013	Watson
2013/0274117	A1	10/2013	Church et al.
2013/0296173	A1	11/2013	Callow et al.
2013/0343317	A1	12/2013	Etemad et al.
2014/0037514	A1	2/2014	Stone et al.
2014/0057799	A1	2/2014	Johnson et al.
2014/0065234	A1	3/2014	Shum et al.
2014/0155295	A1	6/2014	Hindson et al.
2014/0194323	A1	7/2014	Gillevet
2014/0199730	A1	7/2014	Agresti et al.
2014/0199731	A1	7/2014	Agresti et al.
2014/0206554	A1	7/2014	Hindson et al.
2014/0227684	A1	8/2014	Hindson et al.
2014/0227706	A1	8/2014	Kato et al.
2014/0228255	A1	8/2014	Hindson et al.
2014/0235506	A1	8/2014	Hindson et al.
2014/0287963	A1	9/2014	Hindson et al.
2014/0302503	A1	10/2014	Lowe et al.
2014/0378322	A1	12/2014	Hindson et al.
2014/0378345	A1	12/2014	Hindson et al.
2014/0378349	A1	12/2014	Hindson et al.
2014/0378350	A1	12/2014	Hindson et al.
2015/0005199	A1	1/2015	Hindson et al.
2015/0005200	A1	1/2015	Hindson et al.
2015/0011430	A1	1/2015	Saxonov
2015/0011432	A1	1/2015	Saxonov
2015/0111256	A1	4/2015	Church et al.
2015/0119280	A1	4/2015	Srinivas et al.
2015/0218633	A1	8/2015	Hindson et al.
2015/0224466	A1	8/2015	Hindson et al.
2015/0225777	A1	8/2015	Hindson et al.
2015/0292988	A1	10/2015	Bharadwaj et al.
2015/0298091	A1	10/2015	Weitz et al.
2015/0299772	A1	10/2015	Zhang
2015/0376605	A1	12/2015	Jarosz et al.
2015/0376609	A1	12/2015	Hindson et al.
2015/0376700	A1	12/2015	Schnall-Levin et al.
2015/0379196	A1	12/2015	Schnall-Levin et al.
2016/0024558	A1	1/2016	Hardenbol
2016/0122817	A1	5/2016	Jarosz et al.
2016/0244742	A1	8/2016	Linnarsson et al.
2016/0257984	A1	9/2016	Hardenbol et al.
2016/0304860	A1	10/2016	Hindson et al.
2016/0348093	A1	12/2016	Price et al.
2017/0016041	A1	1/2017	Greenfield et al.

FOREIGN PATENT DOCUMENTS

EP	0637996	B1	7/1997
EP	1019496	B1	9/2004
EP	1482036	B1	10/2007
EP	1594980	B1	11/2009
EP	1967592	B1	4/2010
EP	2258846	A2	12/2010
EP	2145955	B1	2/2012
EP	1905828	B1	8/2012
EP	2136786	B1	10/2012
EP	1908832	B1	12/2012
EP	2540389	A1	1/2013
EP	2752664	A1	7/2014
GB	2485850	A	5/2012
JP	S 5949832	A	3/1984
JP	S60227826	A	11/1985
JP	2006-507921	A	3/2006
JP	2006-289250	A	10/2006
JP	2007015990	A	1/2007
JP	2007-268350	A	10/2007
JP	2009-208074	A	9/2009
WO	WO-9530782	A1	11/1995
WO	WO 96/29629	A2	3/1996
WO	WO 96/41011	A1	12/1996
WO	WO 99/09217	A1	2/1999
WO	WO 99/52708	A1	10/1999
WO	WO 00/08212	A1	2/2000
WO	WO 00/26412	A1	5/2000
WO	WO-0070095	A2	11/2000
WO	WO 01/14589	A2	3/2001
WO	WO 01/89787	A2	11/2001
WO	WO 02/31203	A2	4/2002
WO	WO 02/086148	A1	10/2002
WO	WO 2004/002627	A2	1/2004
WO	WO 2004/010106	A2	1/2004
WO	WO-2004069849	A2	8/2004
WO	WO 2004/091763	A2	10/2004
WO	WO 2004/102204	A1	11/2004
WO	WO 2004/103565	A2	12/2004
WO	WO 2004/105734	A1	12/2004
WO	WO-2005002730	A1	1/2005

(56) **References Cited**

FOREIGN PATENT DOCUMENTS

WO	WO 2005/082098	A2	2/2005
WO	WO 2005/021151	A1	3/2005
WO	WO 2005/023331	A2	3/2005
WO	WO 2005/040406	A1	5/2005
WO	WO 2005/049787	A2	6/2005
WO	WO 2006/030993	A1	3/2006
WO	WO 2006/078841	A1	7/2006
WO	WO 2006/096571	A2	9/2006
WO	WO 2007/001448	A2	1/2007
WO	WO 2007/002490	A2	1/2007
WO	WO 2007/024840	A2	3/2007
WO	WO 2007/081385	A2	7/2007
WO	WO 2007/081387	A1	7/2007
WO	WO 2007/089541	A2	8/2007
WO	WO 2007/114794	A1	10/2007
WO	WO 2007/121489	A2	10/2007
WO	WO 2007/133710	A2	11/2007
WO	WO 2007/138178	A2	12/2007
WO	WO 2007/139766	A2	12/2007
WO	WO 2007/140015	A2	12/2007
WO	WO 2007/149432	A2	12/2007
WO	WO 2008/091792	A2	1/2008
WO	WO 2008/021123	A1	2/2008
WO	WO 2008/102057	A1	8/2008
WO	WO 2008/109176	A2	9/2008
WO	WO 2008/121342	A2	10/2008
WO	WO 2008/134153	A1	11/2008
WO	WO 2009/005680	A1	1/2009
WO	WO 2009/011808	A1	1/2009
WO	WO 2009/061372	A1	5/2009
WO	WO 2009/085215	A1	7/2009
WO	WO 2010/004018	A2	1/2010
WO	WO 2010/033200	A2	3/2010
WO	WO-2010104604	A1	9/2010
WO	WO-2010115154	A1	10/2010
WO	WO 2010/148039	A2	12/2010
WO	WO 2010/151776	A2	12/2010
WO	WO 2011/047870	A1	4/2011
WO	WO 2011/056546	A1	5/2011
WO	WO 2011/066476	A1	6/2011
WO	WO-2011074960	A1	6/2011
WO	WO 2012/012037	A1	1/2012
WO	WO 2012/048341	A1	4/2012
WO	WO 2012/083225	A2	6/2012
WO	WO-2012106546	A2	8/2012
WO	WO-2012112970	A2	8/2012
WO	WO-2012142611	A2	10/2012
WO	WO 2012/149042	A2	11/2012
WO	WO 2012/166425	A2	12/2012
WO	WO-2013019751	A1	2/2013
WO	WO-2013036929	A1	3/2013
WO	WO-2013122996	A1	8/2013
WO	WO-2013123125	A1	8/2013
WO	WO 2013/177220	A1	11/2013
WO	WO 2014/028537	A1	2/2014
WO	WO-2014074611	A1	5/2014
WO	WO-2014093676	A1	6/2014
WO	WO-2014210353	A2	12/2014
WO	WO-2015044428	A1	4/2015
WO	WO-2015164212	A1	10/2015

OTHER PUBLICATIONS

U.S. Appl. No. 14/624,468, filed Feb. 17, 2015, Hindson et al.

U.S. Appl. No. 14/624,484, filed Feb. 17, 2015, Hindson et al.

Abate et al., Valve-based flow focusing for drog formation. Appl Phys Lett. 2009;94. 3 pages.

Abate, et al. High-throughput injection with microfluidics using picoinjectors. Proc Natl Acad Sci U S A. Nov. 9, 2010;107(45):19163-6. doi: 10.1073/pNas.1006888107. Epub Oct. 20, 2010.

Advisory Action dated Mar. 21, 2014 for U.S. Appl. No. 13/119,470.

Advisory Action dated May 16, 2014 for U.S. Appl. No. 13/503,588.

Advisory Action mailed Nov. 20, 2013 for U.S. Appl. No. 13/139,326.

Agresti, et al. Selection of ribozymes that catalyse multiple-turnover Diels-Alder cycloadditions by using in vitro compartmentalization. Proc Natl Acad Sci U S A. Nov. 8, 2005;102(45):16170-5. Epub Oct. 31, 2005.

Akselband, "Enrichment of slow-growing marine microorganisms from mixed cultures using gel microdrop (GMD) growth assay and fluorescence-activated cell sorting", J. Exp. Marine Bioi., 329: 196-205 (2006).

Akselband, "Rapid mycobacteria drug susceptibility testing using gel microdrop (GMD) growth assay and flow cytometry", J. Microbiol. Methods, 62:181-197 (2005).

Anna et al., "Formation of dispersions using 'flow focusing' in microchannels", Appln. Phys. Letts. 82:3 364 (2003).

Australian Office Action issued Dec. 17, 2013 for Application No. AU 2010315580.

Boone, et al. Plastic advances microfluidic devices. The devices debuted in silicon and glass, but plastic fabrication may make them hugely successful in biotechnology application. Analytical Chemistry. Feb. 2002; 78A-86A.

Braeckmans et al., Scanning the Code. Modern Drug Discovery. 2003:28-32.

Bransky, et al. A microfluidic droplet generator based on a piezoelectric actuator. Lab Chip. Feb. 21, 2009;9(4):516-20. doi: 10.1039/b814810d. Epub Nov. 20, 2008.

Carroll, "The selection of high-producing cell lines using flow cytometry and cell sorting", Exp. Op. Bioi. Therp., 4:11 1821-1829 (2004).

Chaudhary "A rapid method of cloning functioNal variable-region antibody genese in *Escherichia coli* as single-chain imrnunotoxins" Proc. Nat!. Acad. Sci USA 87: 1066-1070 (Feb. 1990).

Chechetkin et al., Sequencing by hybridization with the generic 6-mer oligonucleotide microarray: an advanced scheme for data processing. J Biomol Struct Dyn. Aug. 2000;18(1):83-101.

Chinese Office Action and search report mailed May 23, 2013 for Application No. CN 200880127116.4.

Chinese office action dated Jun. 18, 2012 for CN Application No. 200880127116.4.

Chou, et al. Disposable Microdevices for DNA Analysis and Cell Sorting. Proc. Solid-State Sensor and Actuator Workshop, Hilton Head, SC. Jun. 8-11, 1998; 11-14.

Clausell-Tormos et al., "Droplet-based microfluidic platforms for the encapsulation and screening of mammalian cells and multicellular organisms", Chem. Biol. 15:427-437 (2008).

De Bruin et al., UBS Investment Research. Q-Series®: DNa Sequencing. UBS Securities LLC. Jul. 12, 2007. 15 pages.

Demirci, et al. Single cell epitaxy by acoustic picolitre droplets. Lab Chip. 2007 Sep;7(9):1139-45. Epub Jul. 10, 2007.

Doerr, "The smallest bioreactor", Nature Methods, 2:5 326 (2005).

Dowding, et al. Oil core/polymer shell microcapsules by interNal phase separation from emulsion droplets. II: controlling the release profile of active molecules. Langmuir. Jun. 7, 2005;21(12):5278-84.

Drmanac et al., Sequencing by hybridization (SBH): advantages, achievements, and opportunities. Adv Biochem Eng Biotechnol. 2002;77 :75-101.

Droplet Based Sequencing (slides) dated (Mar. 12, 2008).

Esser-Kahn, et al. Triggered release from polymer capsules. Macromolecules. 2011; 44:5539-5553.

European office action dated Jan. 23, 2012 for Application No. EP 08865992.5.

European office action dated Apr. 5, 2013 for Application No. EP 08865992.5.

European office action dated Aug. 29, 2013 for Application No. EP 08865992.5.

European office action dated Dec. 15, 2010 for EP Application No. 08865992.5.

Fisher, et al. A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. Genome Biol. 2011;12(1):R1. doi: 10.1186/gb-2011-12-1-r1. Epub Jan. 4, 2011.

(56) **References Cited**

OTHER PUBLICATIONS

Freiberg, et al. Polymer microspheres for controlled drug release. Int J Pharm. Sep. 10, 2004;282(1-2):1-18.
Fu, "A micro fabricated fluorescence-activated cell sorter", Nature Biotech., 17:1109-1111 (1997).
Fulton et al., Advanced multiplexed analysis with the FlowMetrix system. Clin Chern. Sep. 1997;43(9): 1749-56.
Garstecki, et al. Formation of monodisperse bubbles in a microfluidic flow-focusing device. Applied Physics Letters. 2004; 85(13):2649-2651. DOI: 10.1063/1.1796526.
Gartner, et al. The Microfluidic Toolbox—examples for fluidic interfaces and standardization concepts. Proc. SPIE 4982, Microfluidics, BioMEMS, and Medical Microsystems, (Jan. 17, 2003); doi: 10.1117/12.479566.
Ghadessy, et al. Directed evolution of polymerase function by compartmentalized self-replication. Proc Natl Acad Sci U S A. Apr. 10, 2001;98(8):4552-7. Epub Mar. 27, 2001.
Hashimshony, et al. CEL-Seq: Single-Cell RNa-Seq by Multiplexed Linear Amplification. Cell Rep. Sep. 27, 2012;2(3):666-73. doi: 10.1016/j.celrep.2012.08.003. Epub Aug. 30, 2012.
He "Selective Encapsulation of Single Cells and Subcellular Organelles into Picoliter-and Femtoliter-Volume Droplets" ANal. Chern 77: 1539-1544 (2005).
Holtze, et al. Biocompatible surfactants for water-in-fluorocarbon emulsions. Lab Chip. Oct. 2008;8(10):1632-9. doi: 10.1039/b806706f. Epub Sep. 2, 2008.
Huebner, "Quantitative detection of protein expression in single cells using droplet microfluidics", Chern. Commun. 1218-1220 (2007).
Hug, et al. Measurement of the number of molecules of a single mRNA species in a complex mRNA preparation. J Theor Biol. Apr. 21, 2003;221(4):615-24.
International Preliminary Report on Patentability dated Mar. 31, 2011 for PCT/US09/005184.
International Preliminary Report on Patentability dated May 10, 2012 for PCT/US2010/054050.
International Preliminary Report on Patentability dated Jun. 30, 2011 for PCT/US2009/006649.
International Preliminary Report on Patentability dated Jul. 1, 2010 for PCT/US2008/013912.
International Preliminary Report on Patentability dated Sep. 17, 2009 for PCT/US2008/003185 mailed Sep. 17, 2009.
International search report and written opinion dated Jan. 12, 2009 for PCT/US2008/003185.
International search report and written opinion dated Jan. 31, 2011 for PCT/US2010/054050.
International search report and written opinion dated Apr. 3, 2009 for PCT/US2008/013912.
International search report and written opinion dated May 14, 2014 for PCT/US2014/015427.
International search report and written opinion dated May 16, 2014 for PCT/US2013/074764.
International search report and written opinion dated Aug. 16, 2010 for PCT/US2009/005184.
International search report and written opinion dated Aug. 20, 2014 for PCT/US2014/015424.
International search report and written opinion dated Oct. 2, 2009 for PCT/US2009/004037.
International search report and written opinion dated Oct. 21, 2009 for PCT/US2009/003389.
International search report and written opinion dated Oct. 29, 2008 for PCT/US2008/008563.
International search report and written opinion dated Dec. 16, 2013 for PCT/US2013/054797.
International search report dated Apr. 22, 2009 for PCT/US2009/000664.
Japanese Final Rejection dated Aug. 5, 2014 for Application No. JP 2012-536941.
Japanese Office Action and mailed Jul. 17, 2013 for Application No. JP 2010-539498.
Japanese Office Action mailed Nov. 19, 2013 for Application No. JP 2012-536941.
Khomiakov a et al., [Analysis of perfect and mismatched DNA duplexes by a generic hexanucleotide microchip]. Mol Bioi (Mosk). Jul.-Aug. 2003;37(4):726-41. Russian. Abstract only.
Kim, et al. Albumin loaded microsphere of amphiphilic poly(ethylene glycol)/ poly(alpha-ester) multiblock copolymer. Eur J Pharm Sci. Nov. 2004;23(3):245-51.
Kim, et al. Fabrication of monodisperse gel shells and functioNal microgels in microfluidic devices. Angew Chem Int Ed Engl. 2007;46(11):1819-22.
Koster et al., "Drop-based microfluidic devices for encapsulation of single cells", Lab on a Chip The Royal Soc. of Chern. 8: 1110-1115 (2008).
Kutyavin, et al. Oligonucleotides containing 2-aminoadenine and 2-thiothymine act as selectively binding complementary agents. Biochemistry. Aug. 27, 1996;35(34):11170-6.
Li, Y., et al., "PEGylated PLGA Nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats," JourNal of Controlled Release, vol. 71, pp. 203-211 (2001).
Liu, et al. Preparation of uniform-sized PLA microcapsules by combining Shirasu porous glass membrane emulsification technique and multiple emulsion-solvent evaporation method. J Control Release. Mar. 2, 2005;103(1):31-43. Epub Dec. 21, 2004.
Liu, et al. Smart thermo-triggered squirting capsules for Nanoparticle delivery. Soft Matter. 2010; 6(16):3759-3763.
Loscertales, I.G., et al., "Micro/Nano Encapsulation via Electrified Coaxial Liquid Jets," Science, vol. 295, pp. 1695-1698 (2002).
Love, "A microengraving method for rapid selection of single cells producing antigen-specific antibodies", Nature Biotech, 24:6 703 (Jun. 2006).
Marcus. Gene method offers diagnostic hope. The Wall Street JourNal. Jul. 11, 2012.
Mazutis, et al. Selective droplet coalescence using microfluidic systems. Lab Chip. Apr. 24, 2012;12(10):1800-6. doi: 10.1039/c21c40121e. Epub Mar. 27, 2012.
Merriman, et al. Progress in ion torrent semiconductor chip based sequencing. Electrophoresis. Dec. 2012;33(23):3397-417. doi: 10.1002/elps.201200424.
Microfluidic ChipShop. Microfluidic product catalogue. Mar. 2005.
Microfluidic ChipShop. Microfluidic product catalogue. Oct. 2009.
Mirzabekov, "DNA Sequencing by Hybridization—a Megasequencing Method and a Diagnostic Tool?" Trends in Biotechnology 12(1): 27-32 (1994).
Mouritzen et al., Single nucleotide polymorphism genotyping using locked nucleic acid (LNa). Expert Rev Mol Diagn. Jan. 2003;3(1):27-38.
Nguyen, et al. In situ hybridization to chromosomes stabilized in gel microdrops. Cytometry. 1995; 21:111-119.
Notice of Allowance dated Jan. 27, 2014 for U.S. Appl. No. 13/139,326.
Oberholzer, et al. Polymerase chain reaction in liposomes. Chem Biol. Oct. 1995;2(10):677-82.
Office action dated Jan. 4, 2010 for U.S. Appl. No. 12/172,186.
Office action dated Feb. 10, 2014 for U.S. Appl. No. 13/503,588.
Office action dated Feb. 28, 2013 for U.S. Appl. No. 13/139,326.
Office action dated Apr. 24, 2013 for U.S. Appl. No. 13/119,470.
Office action dated May 20, 2014 for U.S. Appl. No. 14/172,266.
Office action dated May 20, 2014 for U.S. Appl. No. 14/172,326.
Office action dated May 28, 2013 for U.S. Appl. No. 12/529,926.
Office action dated Jul. 30, 2014 for U.S. Appl. No. 12/809,120.
Office action dated Aug. 6, 2013 for U.S. Appl. No. 13/139,326.
Office action dated Aug. 6, 2014 for U.S. Appl. No. 12/529,926.
Office action dated Sep. 10, 2014 for U.S. Appl. No. 14/250,701.
Office action dated Sep. 17, 2013 for U.S. Appl. No. 13/503,588.
Office action dated Oct. 1, 2012 for U.S. Appl. No. 12/529,926.
Office action dated Dec. 5, 2013 for U.S. Appl. No. 13/119,470.
Office Action mailed Apr. 29, 2014 for EP Application No. 08865992.5.
Office Action mailed Dec. 16, 2013 for CN Application No. 201080055990.9.
Office Action mailed May 23, 2013 for CN Application No. 200880127116.4.

(56) **References Cited**

OTHER PUBLICATIONS

Ogawa, et al. Production and characterization of O/W emulsions containing cationic droplets stabilized by lecithin-chitosan membranes. J Agric Food Chem. Apr. 23, 2003;51(9):2806-12.
Okushima, "Controlled production of monodisperse double emulsions by two-step droplet breakup in microfluidic devices", Langmuir, 20:9905-9908 (2004).
Perez, C., et al., "Poly(lactic acid)-poly(ethylene glycol) Nanoparticles as new carriers for the delivery ofplasmid DNa," JourNal of Controlled Release, vol. 75, pp. 211-224 (2001).
Peters, et al. Accurate whole-genome sequencing and haplotyping from 10 to 20 human cells. Nature. Jul. 11, 2012;487(7406):190-5. doi: 10.1038/Nature11236.
Plunkett, et al. Chymotrypsin responsive hydrogel: application of a disulfide exchange protocol for the preparation of methacrylamide containing peptides. Biomacromolecules. Mar.-Apr. 2005;6(2):632-7.
Ryan, et al. Rapid assay for mycobacterial growth and antibiotic susceptibility using gel microdrop encapsulation. J Clin Microbiol. Jul. 1995;33(7):1720-6.
Schirinzi et al., Combinatorial sequencing-by-hybridization: aNalysis of the NFI gene. Genet Test. 2006 Spring;10(1):8-17.
Schmitt, "Bead-based multiplex genotyping of human papillomaviruses", J. Clinical Microbial., 44:2 504-512 (2006).
Shah, "Fabrication of mono disperse thermosensitive microgels and gel capsules in micro fluidic devices", Soft Matter, 4:2303-2309 (2008).
Simeonov et al., Single nucleotide polymorphism genotyping using short, fluorescently labeled locked nucleic acid (LNa) probes and fluorescence polarization detection. Nucleic Acids Res. Sep. 1, 2002;30(17):e91.
Sorokin et al., DiscrimiNation between perfect and mismatched duplexes with oligonucleotide gel microchips: role of thermodyNamic and kinetic effects during hybridization. J Biomol Struct Dyn. Jun. 2005;22(6):725-34.
Su, et al., Microfluidics-Based Biochips: Technology Issues, Implementation Platforms, and Design-Automation Challenges. IEEE Transactions on Computer-Aided Design of Integrated Circuits and Systems. 2006;25(2):211-23. (Feb. 2006).
Sun et al., Progress in research and application of liquid-phase chip technology. Chinese JourNal Experimental Surgery. May 2005;22(5):639-40.
Tawfik, et al. Man-made cell-like compartments for molecular evolution. Nat Biotechnol. Jul. 1998;16(7):652-6.
Theberge, et al. Microdropelts in microfluidics: an evolving platform for discoveries in chemsitry and biology. Angew Chem Int Ed Engl. Aug. 9, 2010;49(34):5846-68. doi: 10.1002/anie.200906653.
Tubeleviciute, et al. Compartmentalized self-replication (CSR) selection of Thermococcus litoralis Sh1B DNa polymerase for diminished uracil binding. Protein Eng Des Sel. Aug. 2010;23(8):589-97. doi: 10.1093/protein/gzq032. Epub May 31, 2010.
Wang et al., Single nucleotide polymorphism discrimiNation assisted by improved base stacking hybridization using oligonucleotide microarrays. Biotechniques. 2003;35:300-08.
Wang, et al. A novel thermo-induced self-bursting microcapsule with magnetic-targeting property. Chemphyschem. Oct. 5, 2009;10(14):2405-9.
Weaver, "Rapid clonal growth measurements at the single-cell level: gel microdroplets and flow cytometry", Biotechnology, 9:873-877 (1991).
Whitesides, "Soft lithography in biology and biochemistry", Annual Review of Biomedical Engineering, 3:335-373 (2001).
Williams, et al. Amplification of complex gene libraries by emulsion PCR. Nat Methods. Jul. 2006;3(7):545-50.
Woo, et al. G/C-modified oligodeoxynucleotides with selective complementarity: synthesis and hybridization properties. Nucleic Acids Res. Jul. 1, 1996;24(13):2470-5.
Xia, "Soft lithography", Annual Review of Material Science, 28: 153-184 (1998).
Yamamoto, et al. Chemical modification of Ce(IV)/EDTA-base artificial restriction DNa cutter for versatile manipulation of doulbestranded DNa. Nucleic Acids Research. 2007; 35(7):e53.
Zhang, "Combinatorial marking of cells and organelles with reconstituted fluorescent proteins", Cell, 119:137-144 (Oct. 1, 2004).
Zhang, et al. Degradable disulfide core-cross-linked micelles as a drug delivery system prepared from vinyl functioNalized nucleosides via the RAFT process. Biomacromolecules. Nov. 2008;9(11):3321-31. doi: 10.1021/bm800867n. Epub Oct. 9, 2008.
Zhao, J., et al., "Preparation of hemoglobin-loaded Nano-sized particles with porous structure as oxygen carriers," Biomaterials, vol. 28, pp. 1414-1422 (2007).
Zimmermann et at., Microscale production of hybridomas by hypoosmolar electrofusion. Hum• Antibodies Hybridomas. Jan. 1992;3(1): 14-8.
Zong, et al. Genome-wide detection of single-nucleotide and copy-number variations of a single human cell. Science. Dec. 21, 2012;338(6114):1622-6. doi: 10.1126/science.1229164.
Office action dated Jan. 15, 2015 for U.S. Appl. No. 14/250,701.
International search report and written opinion dated May 14, 2015 for PCT/US2014/044398.
International search report and written opinion dated Aug. 19, 2015 for PCT/US2015/025197.
Office action dated Sep. 16, 2015 for U.S. Appl. No. 14/175,973.
Office action dated Oct. 9, 2015 for US Appl. No. 14/680,808.
Office action dated Feb. 23, 2016 for U.S. Appl. No. 14/104,650.
Office action dated Mar. 1, 2016 for U.S. Appl. No. 14/250,701.
Office action dated Mar. 4, 2016 for U.S. Appl. No. 14/175,973.
Office action dated Mar. 14, 2016 for U.S. Appl. No. 14/680,808.
Shimkus, et al. A chemically cleavable biotinylated nucleotide: usefulness in the recovery of protein-DNA complexes from avidin affinity columns. Proc Natl Acad Sci U S A. May 1985;82(9):2593-7.
U.S. Appl. No. 14/682,952, filed Apr. 8, 2015, Bharadwaj et al.
Gyarmati, et al. Reversible disulphide formation in polymer networks: a versatile functional group from synthesis to applications. European Polymer Journal. 2013; 49:1268-1286.
Office action dated Apr. 20, 2015 for U.S. Appl. No. 13/966,150.
Office action dated Sep. 25, 2015 for U.S. Appl. No. 14/250,701.
European search report and opinion dated Feb. 2, 2016 for EP Application No. 13829413.
Office action dated Jan. 29, 2016 for U.S. Appl. No. 14/175,935.
Office action dated Nov. 6, 2015 for U.S. Appl. No. 13/966,150.
Berkum, et al. Hi-C: a method to study the three-dimensional architecture of genomes. J Vis Exp. May 6, 2010;(39). pii: 1869. doi: 10.3791/1869.
Chung, et al. Structural and molecular interrogation of intact biological systems. Nature. May 16, 2013;497(7449):332-7. doi: 10.1038/nature12107. Epub Apr. 10, 2013.
Dekker, et al. Capturing chromosome conformation. Science. Feb. 15, 2002;295(5558):1306-11.
Illumina Nextera Enrichment Sample Preparation Guide. Feb. 2013.
Illumina TruSeq Custom Enrichment Kit Data Sheet. (c) 2014.
Kaper, et al. Supporting Information for "Whole-genome haplotyping by dilution, amplification, and sequencing." Proc Natl Acad Sci U S A. Apr. 2, 2013;110(14):5552-7. doi: 10.1073/pnas.1218696110. Epub Mar. 18, 2013.
Kaper, et al. Whole-genome haplotyping by dilution, amplification, and sequencing. Proc Natl Acad Sci U S A. Apr. 2, 2013;110(14):5552-7. doi: 10.1073/pnas.1218696110. Epub Mar. 18, 2013.
Karmakar, et al. Organocatalytic removal of formaldehyde adducts from RNA and DNA bases. Nat Chem. Sep. 2015;7(9):752-8. doi: 10.1038/nchem.2307. Epub Aug. 3, 2015.
Kebschull, et al. High-Throughput Mapping of Single-Neuron Projections by Sequencing of Barcoded RNA. Neuron. Sep. 7, 2016;91(5):975-87. doi: 10.1016/j.neuron.2016.07.036. Epub Aug. 18, 2016.
Lee, et al. Act-Presto: Rapid and consistent tissue clearing and labeling method for 3-dimensional (3D) imaging. Sci Rep. Jan. 11, 2016;6:18631. doi: 10.1038/srep18631.

(56) **References Cited**

OTHER PUBLICATIONS

Susaki, et al. Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. Cell. Apr. 24, 2014;157(3):726-39. doi: 10.1016/j.cell.2014.03.042. Epub Apr. 17, 2014.

Tomer, et al. Advanced Clarity for rapid and high-resolution imaging of intact tissues. Nat Protoc. Jul. 2014;9(7):1682-97. doi: 10.1038/nprot.2014.123. Epub Jun. 19, 2014.

Wesolowska, et al. Cost-effective multiplexing before capture allows screening of 25 000 clinically relevant SNPs in childhood acute lymphoblastic leukemia. Leukemia. Jun. 2011;25(6):1001-6. doi: 10.1038/leu.2011.32. Epub Mar. 18, 2011.

Abate, et al. Beating Poisson encapsulation statistics using close-packed ordering. Lab Chip. Sep. 21, 2009;9(18):2628-31. doi: 10.1039/b909386a. Epub Jul. 28, 2009.

Altemos et al., "Genomic Characterization of Large Heterochromatic Gaps in the Human Genome Assembly," PLOS Computational Biology, May 15, 2014, vol. 10, Issue 5, 14 pages.

Amini, S. et al. "Haplotype-resolved whole-genome sequencing by contiguity-preserving transposition and combinatorial indexing" Nature Genetics (2014) 46:1343-1349 doi:10.1038/ng.3119.

Anonymous, "Oligo(dT)25 cellulose beads" NEB (2012) Retrieved from the Internet:https://www.neb.com/~/media/Catalog/All-Products/286CA51268E24DE1B06F1CB288698B54/Datacards%20or%Manuals/S1408Datasheet-Lot0011205.pdf.

Anonymous, "Oligotex Handbook" Qiagen (2012) XP055314680, Retrieved from the Internet: URL:http://www.qiagen.com/de/resources/download.apsx?id=f9fald98-d54d-47e7-a20b-8b0cb8975009&lang=en.

Anonymous: "Viscosity-Basic concepts" (2004) XP055314117, Retrieved from the Internet: URL:http://lhtc.epfl.ch/webdav/site/lhtc/shared/import/migration/2 VISCOSITY.pdf.

Attia, et al. Micro-injection moulding of polymer microfluidic devices. Microfluidics and nanofluidics. 2009; 7(1):1-28.

Baret, et al. Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. Lab Chip. Jul. 7, 2009;9(13):1850-8. doi: 10.1039/b902504a. Epub Apr. 23, 2009.

Bodi, K. et al. "Comparison of Commercially Available Target Enrichment Methods for Next-Generation Sequencing" J Biomolecular Techniques (2013) 24:73-86.

Brouzes, et al. Droplet microfluidic technology for single-cell high-throughput screening. Proc Natl Acad Sci U S A. Aug. 25, 2009;106(34):14195-200. doi: 10.1073/pnas.0903542106. Epub Jul. 15, 2009.

Chen, et al. Chemical transfection of cells in picoliter aqueous droplets in fluorocarbon oil. Anal Chem. Nov. 15, 2011;83(22):8816-20. doi: 10.1021/ac2022794. Epub Oct. 17, 2011.

Chokkalingam, et al. Probing cellular heterogeneity in cytokine-secreting immune cells using droplet-based microfluidics. Lab Chip. Dec. 21, 2013;13(24):4740-4. doi: 10.1039/c3lc50945a.

Christiansen et al. "The Covalent Eukaryotic Topoisomerase I-DNA Intermediate Catalyzes pH-dependent Hydrolysis and Alcholysis" J Biol Chem (Apr. 14, 1994) 269(15):11367-11373.

Chu, et al. Controllable monodisperse multiple emulsions. Angew Chem Int Ed Engl. 2007;46(47):8970-4.

Coufal, et al. L1 retrotransposition in human neural progenitor cells. Nature. Aug. 27, 2009;460(7259):1127-31. doi: 10.1038/nature08248. Epub Aug. 5, 2009.

Doshi, et al. Red blood cell-mimicking synthetic biomaterial particles. Proceedings of the National Academy of Sciences 106.51 (2009): 21495-21499.

Draper, et al. Compartmentalization of electrophoretically separated analytes in a multiphase microfluidic platform. Anal Chem. Jul. 3, 2012;84(13):5801-8. doi: 10.1021/ac301141x. Epub Jun. 13, 2012.

Dressler, et al. Droplet-based microfluidics enabling impact on drug discovery. J Biomol Screen. Apr. 2014;19(4):483-96. doi: 10.1177/1087057113510401. Epub Nov. 15, 2013.

"Eastburn, et al. Ultrahigh-throughput mammalian single-cell reverse-transcriptase polymerase chain reaction in microfluidic droplets. Anal Chem. Aug. 20, 2013;85(16):8016-21. doi: 10.1021/ac402057q. Epub Aug. 8, 2013."

Fan, et al. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci U S A. Oct. 21, 2008;105(42):16266-71. doi: 10.1073/pnas.0808319105. Epub Oct. 6, 2008.

Fang, et al. Fluoride-cleavable biotinylation phosphoramidite for 5'-end-labeling and affinity purification of synthetic oligonucleotides. Nucleic Acids Res. Jan. 15, 2003;31(2):708-15.

Frampton, G.M. et al. "Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing" Nature Biotechnology (2013) 31(11):1023-1031. doi:10.1038/nbr.2696.

Fredrickson, et al. Macro-to-micro interfaces for microfluidic devices. Lab Chip. Dec. 2004;4(6):526-33. Epub Nov. 10, 2004.

Fu, A.Y., et al., "A microfabricated fluorescence-activated cell sorter," Nature Biotechnology, vol. 17, pp. 1109-1111 (1999).

Gericke, et al. Functional cellulose beads: preparation, characterization, and applications. Chemical reviews 113.7 (2013): 4812-4836.

Granieri, Lucia. Droplet-based microfluidics and engineering of tissue plasminogen activator for biomedical applications. Ph.D. Thesis, Nov. 13, 2009 (131 pages).

Grasland-Mongrain, et al. Droplet coalescence in microfluidic devices. Jan.-Jul. 2003. 31 pages. http://www.eleves.ens.fr/home/grasland/rapports/stage4.pdf.

Guo, et al. Droplet microfluidics for high-throughput biological assays. Lab Chip. Jun. 21, 2012;12(12):2146-55. doi: 10.1039/c21c21147e. Epub Feb. 9, 2012.

He, J. et al. "Genotyping-by-sequencing (GBS), an ultimate marker-assisted selections (MAS) tool to accelerate plant breeding" Frontiers in Plant Sci (Sep. 30, 2014) 5:1-8.

Hjerten, et al. General methods to render macroporous stationary phases nonporous and deformable, exemplified with agarose and silica beads and their use in high-performance ion-exchange and hydrophobic-interaction chromatography of proteins. Chromatographia 31.1-2 (1991): 85-94.

Ioannidis, N. Manufacturing of Agarose-Based Chromatographic Adsorbents With Controlled Pore and Particle Sizes. A thesis submitted to The University of Birmingham for the degree of Doctor of Philosophy. 2009.

Jena, et al. Cyclic olefin copolymer based microfluidic devices for biochip applications: Ultraviolet surface grafting using 2-methacryloyloxyethyl phosphorylcholine. Biomicrofluidics. Mar. 2012;6(1):12822-1282212. doi: 10.1063/1.3682098. Epub Mar. 15, 2012.

Jung, et al. Micro machining of injection mold inserts for fluidic channel of polymeric biochips. Sensors. 2007; 7(8):1643-1654.

Kim, et al. Rapid prototyping of microfluidic systems using a PDMS/polymer tape composite. Lab Chip. May 7, 2009;9(9):1290-3. doi: 10.1039/b818389a. Epub Feb. 10, 2009.

Klein, et al. Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. Cell. May 21, 2015;161(5):1187-201. doi: 10.1016/j.ce11.2015.04.044.

Lagus, et al. A review of the theory, methods and recent applications of high-throughput single-cell droplet microfluidics. J. Phys. D: Appl. Phys. (2013) 46:114005. (21 pages).

Lee, J-H. et al. "Fluorescent in situ sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues" Nature Protocols (Feb. 12, 2015) 10(3):442-458.

Lowe, Adam J. Norbornenes and [n]polynorbornanes as molecular scaffolds for anion recognition. Ph.D. Thesis (May 2010). (361 pages).

Macosko, et al. Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. Cell. May 21, 2015;161(5)1202-14. doi: 10.1016/j.ce11.2015.05.002.

Mair, et al. Injection molded microfluidic chips featuring integrated interconnects. Lab Chip. Oct. 2006;6(10):1346-54. Epub Jul. 31, 2006.

(56) **References Cited**

OTHER PUBLICATIONS

Makino, et al. Preparation of hydrogel microcapsules: Effects of preparation conditions upon membrane properties. Colloids and Surfaces B: Biointerfaces. Nov. 1998; 12(2), 97-104.
Matochko, et al. Uniform amplification of phage display libraries in monodisperse emulsions. Methods. Sep. 2012;58(1):18-27. doi: 10.1016/j.ymeth.2012.07.012. Epub Jul. 20, 2012.
Moore, et al. Behavior of capillary valves in centrifugal microfluidic devices prepared by three-dimensional printing. Microfluidics and Nanofluidics. 2011; 10(4):877-888.
Morgan, et al. Chapter 12: Human microbiome analysis. PLoS Comput Biol. 2012;8(12):e1002808. doi: 10.1371/journal.pcbi.1002808. Epub Dec. 27, 2012.
Mozhanova, A.A. et al. "Local elastic properties of biological materials studied by SFM" (2003) XP055314108, Retrieved from the Internet: URL:http://www.ntmdt.com/data/media/files/publications/2003/08.08_a.a.mozhanova_nin_english.pdf.
Muotri, et al. L1 retrotransposition in neurons is modulated by MeCP2. Nature. Nov. 18, 2010;468(7322):443-6. doi: 10.1038/nature09544.
Nagashima, et al. Preparation of monodisperse poly (acrylamide-co-acrylic acid) hydrogel microspheres by a membrane emulsification technique and their size-dependent surface properties. Colloids and Surfaces B: Biointerfaces. Jun. 15, 1998; 11(1-2), 47-56.
Navin. The first five years of single-cell cancer genomics and beyond. Genome Res. Oct. 2015;25(10):1499-507. doi: 10.1101/gr.191098.115.
Novak, et al. Single cell multiplex gene detection and sequencing using microfluidicallygenerated agarose emulsions. Angew Chem Int Ed Engl. Jan. 10, 2011;50(2):390-5. doi: 10.1002/anie.201006089.
Oligotex Handbook. For purification of poly A+ RNA from total RNA and directly from cultured cells or tissues as well as purification of polyadenylated in vitro transcripts. Jun. 2012.
Pantel, et al. Detection methods of circulating tumor cells. J Thorac Dis. Oct. 2012;4(5):446-7. doi: 10.3978/j.issn.2072-1439.2012.08.15.
Patel, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. Science. Jun. 20, 2014;244(6190):1396-401. doi: 10.1126/science.1254257. Epub Jun. 12, 2014.
Picot, J. et al. "A biomimetic microfluidic chip to study the circulation and mechanical retention of red blood cells in the spleen" Am J Hematology (Jan. 12, 2015) 90(4):339-345.
Ram, et al. Strategy for microbiome analysis using 16S rRNA gene sequence analysis on the Illumina sequencing platform. Syst Biol Reprod Med. Jun. 2011;57(3):162-70. doi: 10.3109/19396368.2011.555598. Epub Mar. 1, 2011.
Richardson, et al. Novel inhibition of archaeal family-D DNA polymerase by uracil. Nucleic acids research 41.7 (2013): 4207-4218.
Rogozin, et al. A highly conserved family of inactivated archaeal B family DNA polymerases. Biol Direct. Aug. 6, 2008;3:32. doi: 10.1186/1745-6150-3-32.
Rotem, et al. High-Throughput Single-Cell Labeling (Hi-SCL) for RNA-Seq Using Drop-Based Microfluidics. PLoS One. May 22, 2015;10(5):e0116328. doi: 10.1371/journal.pone.0116328. eCollection 2015.
Rotem, et al. Single Cell Chip-Seq Using Drop-Based Microfluidics. Abstract #50. Frontiers of Single Cell Analysis, Stanford University Sep. 5-7, 2013.
Schmeider, et al. Fast identification and removal of sequence contamination from genomic and metagenomic datasets. PLoS One. Mar. 9, 2011;6(3):e17288. doi: 10.1371/journal.pone.0017288.
Seiffert, et al. Smart microgel capsules from macromolecular precursors. J Am Chem Soc. May 12, 2010;132(18):6606-9. doi: 10.1021/ja102156h.
Shuttleworth, et al. Recognition of the pro-mutagenic base uracil by family B DNA polymerases from archaea. J Mol Biol. Mar. 26, 2004;337(3):621-34.
Tayyab, S. et al. "Size exclusion chromatography and size exclusion HPLC of proteins" Biochem Ed, Pergamon, (1991) 19(3):149-152.
Tewhey, et al. Microdroplet-based PCR enrichment for large-scale targeted sequencing. Nat Biotechnol. Nov. 2009;27(11):1025-31. doi: 10.1038/nbt.1583. Epub Nov. 1, 2009.
Tonelli, et al. Perfluoropolyether functional oligomers: unusual reactivity in organic chemistry. Journal of fluorine chemistry. 2002; 118(1)"107-121.
Turner, et al. Methods for genomic partitioning. Annu Rev Genomics Hum Genet. 2009;10:263-84. doi: 10.1146/annurev-genom-082908-150112. Review.
Wagner, et al. Biocompatible fluorinated polyglycerols for droplet microfluidics as an alternative to PEG-based copolymer surfactants. Lab Chip. Jan. 7, 2016;16(1):65-9. doi: 10.1039/c5lc00823a. Epub Dec. 2, 2015.
Zheng, X.Y. et al. "Haplotyping germline and cancer genomes with high-throughput linked-read sequencing" Nature Biotech (Feb. 1, 2016) 34(3):303-311.
Zhu, et al. Synthesis and self-assembly of highly incompatible polybutadienepoly(hexafluoropropoylene oxide) diblock copolymers. Journal of Polymer Science Part B: Polymer Physics. 2005; 43(24):3685-3694.
Aitman, et al. Copy number polymorphism in Fcgr3 predisposes to glomerulonephritis in rats and humans. Nature. Feb. 16, 2006;439(7078):851-5.
Balikova, et al. Autosomal-dominant microtia linked to five tandem copies of a copy-number-variable region at chromosome 4p16. Am J Hum Genet. Jan. 2008;82(1):181-7. doi: 10.1016/j.ajhg.2007.08.001.
Browning, et al. Haplotype phasing: existing methods and new developments. Nat Rev Genet. Sep. 16, 2011;12(10):703-14. doi: 10.1038/nrg3054. Review.
Cappuzzo, et al. Increased HER2 gene copy number is associated with response to gefitinib therapy in epidermal growth factor receptor-positive non-small-cell lung cancer patients. J Clin Oncol. Aug. 1, 2005;23(22):5007-18.
Choi, et al. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. Cancer Res. Jul. 1, 2008;68(13):4971-6. doi: 10.1158/0008-5472.CAN-07-6158.
Cook, et al. Copy-number variations associated with neuropsychiatric conditions. Nature. Oct. 16, 2008;455(7215):919-23. doi: 10.1038/nature07458.
Co-pending U.S. Appl. No. 15/392,557, filed Dec. 28, 2016.
"Dressman et al. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. Proc. Natl. Acad. Sci. 2003. 100(15):8817-8822."
Fabi, et al. Correlation of efficacy between EGFR gene copy number and lapatinib/capecitabine therapy in HER2-positive metastatic breast cancer. J. Clin. Oncol. 2010; 28:15S. 2010 ASCO Meeting abstract Jun. 14, 2010:1059.
Gonzalez, et al. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science. Mar. 4, 2005;307(5714):1434-40. Epub Jan. 6, 2005.
"Sigma. Streptavidin-agarose (S1638) product information sheet. www.sigma-aldrich.com".
Kitzman, et al. Noninvasive whole-genome sequencing of a human fetus. Sci Transl Med. Jun. 6, 2012;4(137):137ra76. doi: 10.1126/scitranslmed.3004323.
Knight, et al. Subtle chromosomal rearrangements in children with unexplained mental retardation. Lancet. Nov. 13, 1999;354(9191):1676-81.
Lupski. Genomic rearrangements and sporadic disease. Nat Genet. Jul. 2007;39(7 Suppl):S43-7.
Pinto, et al. Functional impact of global rare copy number variation in autism spectrum disorders. Nature. Jul. 15, 2010;466(7304):368-72. doi: 10.1038/nature09146. Epub Jun. 9, 2010.
Ropers. New perspectives for the elucidation of genetic disorders. Am J Hum Genet. Aug. 2007;81(2):199-207. Epub Jun. 29, 2007.
Sebat, et al. Strong association of de novo copy number mutations with autism. Science. Apr. 20, 2007;316(5823):445-9. Epub Mar. 15, 2007.
Shlien, et al. Copy number variations and cancer. Genome Med. Jun. 16, 2009;1(6):62. doi: 10.1186/gm62.

(56) **References Cited**

OTHER PUBLICATIONS

Shlien, et al. Excessive genomic DNA copy number variation in the Li-Fraumeni cancer predisposition syndrome. Proc Natl Acad Sci U S A. Aug. 12, 2008;105(32):11264-9. doi: 10.1073/pnas.0802970105. Epub Aug. 6, 2008.
Wang, et al. Digital karyotyping. Proc Natl Acad Sci U S A. Dec. 10, 2002;99(25):16156-61. Epub Dec. 2, 2002.
Co-pending U.S. Appl. No. 15/440,772, filed Feb. 23, 2017.
Co-pending U.S. Appl. No. 15/449,741, filed Mar. 3, 2017.
U.S. Appl. No. 14/680,808, filed Apr. 7, 2015, Hindson et al.
Co-pending U.S. Appl. No. 15/430,298, filed Feb. 10, 2017.
Co-pending U.S. Appl. No. 15/470,814, filed Mar. 27, 2017.
Co-pending U.S. Appl. No. 15/598,898, filed May 18, 2017.
Stoeckius, et al. Large-scale simultaneous measurement of epitopes and transcriptomes in single cells. bioRxiv 113068; doi: https://doi.org/10.1101/113068.

* cited by examiner

Fig. 1A

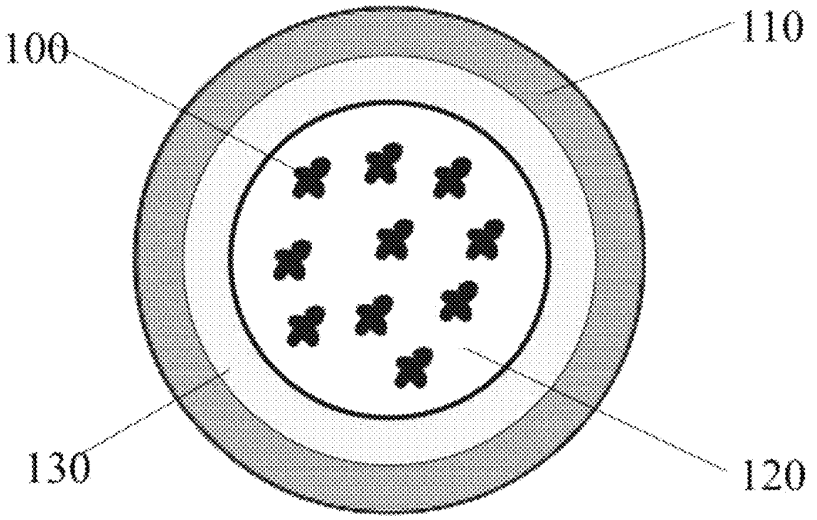


Fig. 1B

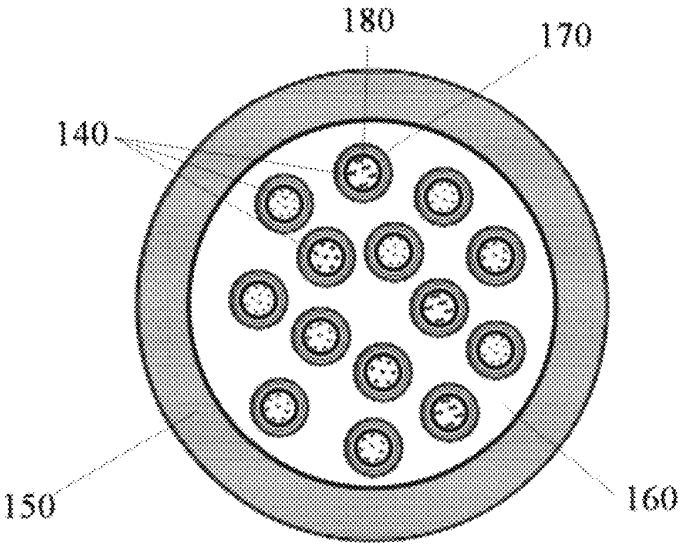


Fig. 2A

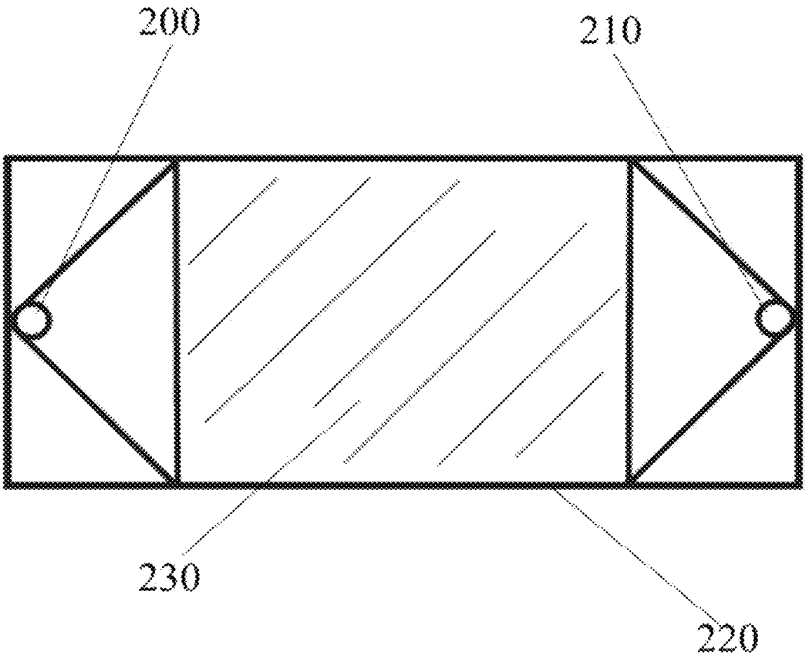


Fig. 2B

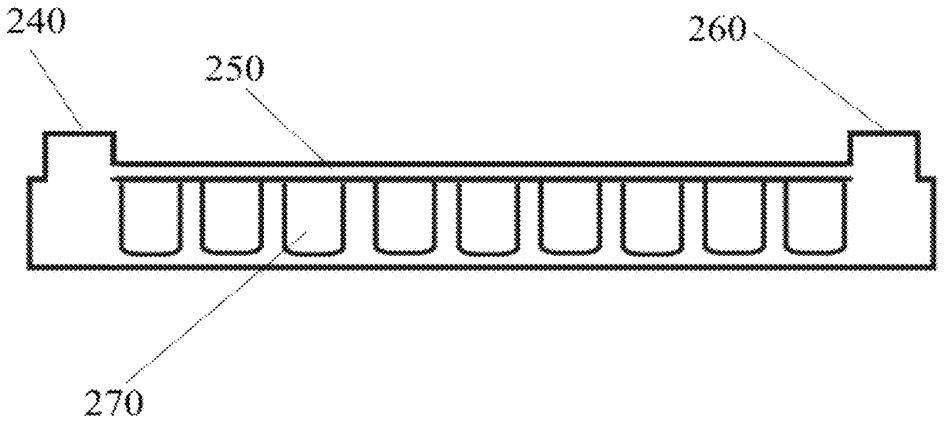


Fig. 3

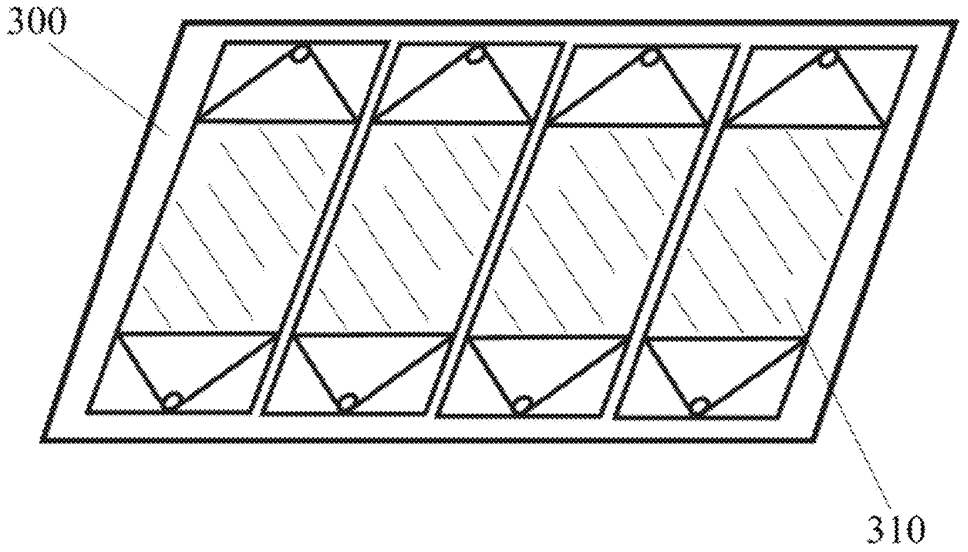


Fig. 4A

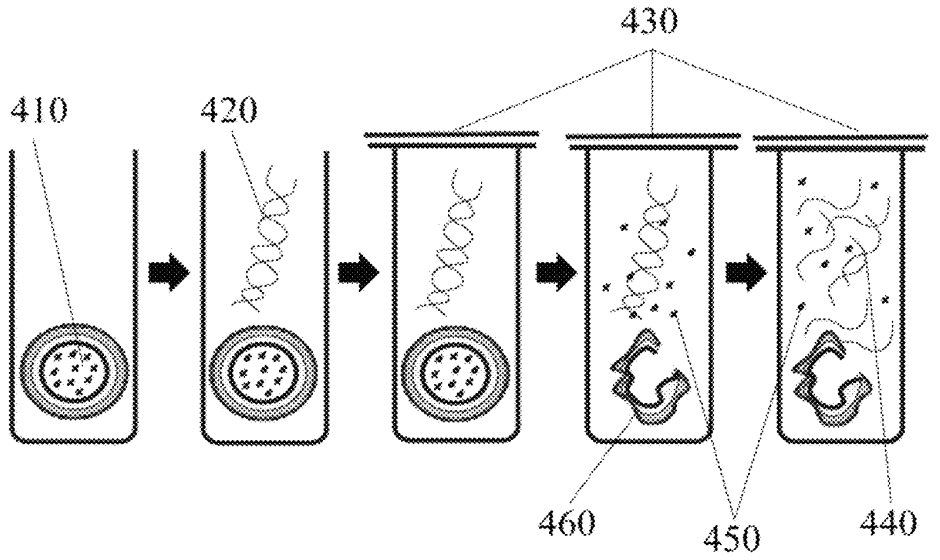
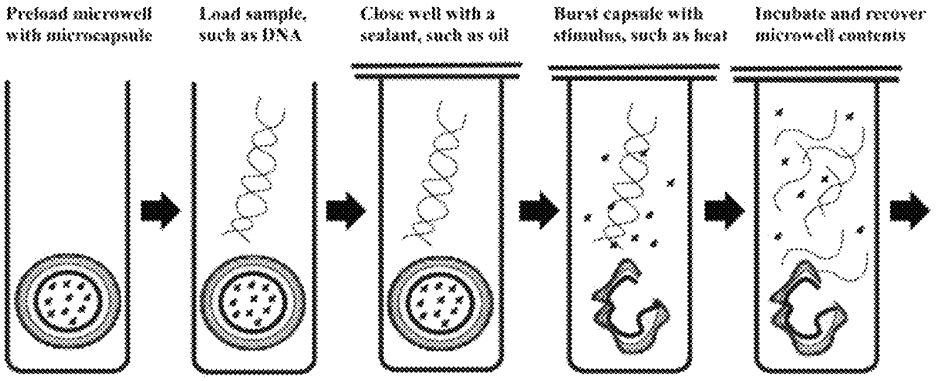

430
410
420
460
450
440

Fig. 4B


Preload microwell with microcapsule
Load sample, such as DNA
Close well with a sealant, such as oil
Burst capsule with stimulus, such as heat
Incubate and recover microwell contents

METHODS FOR DROPLET-BASED SAMPLE PREPARATION

CROSS-REFERENCE

This application is a continuation of U.S. patent application Ser. No. 13/966,150, filed Aug. 13, 2013, which applications claim the benefit of U.S. Provisional Patent Application No. 61/683,192, filed Aug. 14, 2012; U.S. Provisional Patent Application No. 61/737,374, filed Dec. 14, 2012; U.S. Provisional Patent Application No. 61/762,435, filed Feb. 8, 2013; U.S. Provisional Patent Application No. 61/800,223, filed Mar. 15, 2013; U.S. Provisional Patent Application No. 61/840,403, filed Jun. 27, 2013; and U.S. Provisional Patent Application No. 61/844,804, filed Jul. 10, 2013, which applications are incorporated herein by reference in their entireties for all purposes.

BACKGROUND OF THE INVENTION

The detection and quantification of analytes is important for molecular biology and medical applications such as diagnostics. Genetic testing is particularly useful for a number of diagnostic methods. For example, disorders that are caused by mutations, such as cancer, may be detected or more accurately characterized with DNA sequence information.

Appropriate sample preparation is often needed prior to conducting a molecular reaction such as a sequencing reaction. A starting sample may be a biological sample such as a collection of cells, tissue, or nucleic acids. When the starting material is cells or tissue, the sample may need to be lysed or otherwise manipulated in order to permit the extraction of molecules such as DNA. Sample preparation may also involve fragmenting molecules, isolating molecules, and/or attaching unique identifiers to particular fragments of molecules, among other actions. There is a need in the art for improved methods and devices for preparing samples prior to downstream applications.

SUMMARY OF THE INVENTION

This disclosure provides compositions and methods for a microcapsule array device.

An aspect of the disclosure provides a composition comprising a first microcapsule, wherein: the first microcapsule is degradable upon the application of a stimulus to the first microcapsule; and the first microcapsule comprises an oligonucleotide barcode. In some cases, the first microcapsule may comprise a chemical cross-linker. The chemical cross-linker, for example, may be a disulfide bond. In some cases, the composition may comprise a polymer gel, such as, for example a polyacrylamide gel. The first microcapsule may comprise a bead. In some cases, the bead may be a gel bead.

Moreover, the stimulus may be selected from the group consisting of a biological, chemical, thermal, electrical, magnetic, or photo stimulus, and combination thereof. In some cases, the chemical stimulus may be selected from the group consisting of a change in pH, a change in ion concentration, and a reducing agent. The reducing agent may be, for example, dithiothreitol (DTT) or tris(2-carboxyethyl) phosphine (TCEP).

A second microcapsule may comprise the first microcapsule. Moreover, the second microcapsule may be a droplet. In some cases, the composition may also comprise a nucleic acid that comprises the oligonucleotide barcode, wherein the nucleic acid comprises a deoxyuridine triphosphate (dUTP).

In some cases, the composition may comprise a polymerase unable to accept a deoxyuridine triphosphate (dUTP). Also, the composition may comprise a target analyte, such as, for example, a nucleic acid. The nucleic acid may be selected from the group consisting of DNA, RNA, dNTPs, ddNTPs, amplicons, synthetic nucleotides, synthetic polynucleotides, polynucleotides, oligonucleotides, peptide nucleic acids, cDNA, dsDNA, ssDNA, plasmid DNA, cosmid DNA, High Molecular Weight (MW) DNA, chromosomal DNA, genomic DNA, viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), mRNA, rRNA, tRNA, nRNA, siRNA, snRNA, snoRNA, scaRNA, microRNA, dsRNA, ribozyme, riboswitch and viral RNA. In some cases, the nucleic acid may be genomic DNA (gDNA).

Additionally, the density of the oligonucleotide barcodes may be at least about 1,000,000 oligonucleotide barcodes per the first microcapsule. The oligonucleotide barcode may be coupled to the microcapsule via a chemical cross-linker, such as, for example a disulfide bond.

An additional aspect of the disclosure comprises a device comprising a plurality of partitions, wherein: at least one partition of the plurality of partitions comprises a microcapsule comprising an oligonucleotide barcode; and the microcapsule is degradable upon the application of a stimulus to the microcapsule. The partition, for example, may be a well or a droplet. In some cases, the microcapsule comprises a chemical cross-linker such as, for example, a disulfide bond. Moreover, the microcapsule may comprise a polymer gel such as, for example, a polyacrylamide gel. Also, the microcapsule may comprise a bead. In some cases, the bead may be a gel bead.

The stimulus may be selected from the group consisting of a biological, chemical, thermal, electrical, magnetic, or photo stimulus, and a combination thereof. In some cases, the chemical stimulus may be selected from the group consisting of a change in pH, change in ion concentration, and a reducing agent. The reducing agent, for example, may be dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP).

Furthermore, a nucleic acid may comprise the oligonucleotide barcode and the nucleic acid may comprise a deoxyuridine triphosphate (dUTP). In some cases, the partition may comprise a polymerase unable to accept a deoxyuridine triphosphate (dUTP). Additionally, the partition may comprise a target analyte such as, for example, a nucleic acid. The nucleic acid may be selected from the group consisting of DNA, RNA, dNTPs, ddNTPs, amplicons, synthetic nucleotides, synthetic polynucleotides, polynucleotides, oligonucleotides, peptide nucleic acids, cDNA, dsDNA, ssDNA, plasmid DNA, cosmid DNA, High Molecular Weight (MW) DNA, chromosomal DNA, genomic DNA, viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), mRNA, rRNA, tRNA, nRNA, siRNA, snRNA, snoRNA, scaRNA, microRNA, dsRNA, ribozyme, riboswitch and viral RNA. In some cases, the nucleic acid may be genomic DNA (gDNA). The oligonucleotide barcode may be coupled to the microcapsule via a chemical cross-linker. In some cases, the chemical cross-linker may be a disulfide bond.

A further aspect of the disclosure provides a method for sample preparation comprising combining a microcapsule comprising an oligonucleotide barcode and a target analyte into a partition, wherein the microcapsule is degradable upon the application of a stimulus to the microcapsule; and applying the stimulus to the microcapsule to release the oligonucleotide barcode to the target analyte. The partition may be, for example, a well or a droplet. In some cases, the microcapsule may comprise a polymer gel such as, for

example, a polyacrylamide. Moreover, the microcapsule may comprise a bead. In some cases, the bead may be a gel bead. Moreover, the microcapsule may comprise a chemical cross-linker such as, for example, a disulfide bond.

The stimulus may be selected from the group consisting of a biological, chemical, thermal, electrical, magnetic, photo stimulus, and a combination thereof. In some cases, the chemical stimulus may be selected from the group consisting of a change in pH, change in ion concentration, and a reducing agent. The reducing agent may be, for example, dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP).

Also, a nucleic acid may comprise the oligonucleotide barcode and the nucleic acid may comprise a deoxyuridine triphosphate (dUTP). In some cases, the partition may comprise a polymerase unable to accept a deoxyuridine triphosphate (dUTP). Moreover, the method may also comprise attaching the oligonucleotide barcode to the target analyte. The attaching may be completed, for example, with a nucleic acid amplification reaction. Moreover, the analyte may be a nucleic acid. In some cases, the nucleic acid may be selected from the group consisting of DNA, RNA, dNTPs, ddNTPs, amplicons, synthetic nucleotides, synthetic polynucleotides, polynucleotides, oligonucleotides, peptide nucleic acids, cDNA, dsDNA, ssDNA, plasmid DNA, cosmid DNA, High Molecular Weight (MW) DNA, chromosomal DNA, genomic DNA, viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), mRNA, rRNA, tRNA, nRNA, siRNA, snRNA, snoRNA, scaRNA, microRNA, dsRNA, ribozyme, riboswitch and viral RNA. In some cases, the nucleic acid may be genomic DNA (gDNA). Furthermore, the oligonucleotide barcode may be coupled to the microcapsule via a chemical cross-linker. In some cases, the chemical cross-linker may be a disulfide bond.

A further aspect of the disclosure provides a composition comprising a degradable gel bead, wherein the gel bead comprises at least about 1,000,000 oligonucleotide barcodes. In some cases, the 1,000,000 oligonucleotide barcodes are identical.

INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference in their entireties for all purposes and to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

The novel features of a device of this disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of this disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of a device of this disclosure are utilized, and the accompanying drawings of which:

FIG. **1**A is a schematic representation of a microcapsule or inner reagent droplet.

FIG. **1**B is a schematic representation of a microcapsule containing multiple inner reagent droplets.

FIG. **2**A is a schematic illustration of a top down view of an exemplary microcapsule array.

FIG. **2**B is a schematic illustration of an exemplary side view of a microcapsule array.

FIG. **3** is a schematic illustration of a multi-microcapsule array configuration on a 96-well plate holder.

FIG. **4**A is a schematic flow diagram representative of a reaction sequence in one microwell of a microwell capsule array.

FIG. **4**B is similar to **4**A, except that it is annotated with examples of methods that can be performed at each step.

DETAILED DESCRIPTION OF THE INVENTION

While various embodiments of the invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions may occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed.

I. General Overview

The present disclosure provides microwell or other partition capsule array devices and methods of using such devices. Generally, the device is an assembly of partitions (e.g., microwells, droplets) that are loaded with microcapsules, often at a particular concentration of microcapsules per partition.

The devices may be particularly useful to perform sample preparation operations. In some cases, a device subdivides a sample (e.g., a heterogeneous mixture of nucleic acids, a mixture of cells, etc.) into multiple partitions such that only a portion of the sample is present in each partition. For example, a nucleic acid sample comprising a mixture of nucleic acids may be partitioned such that no more than one strand of (or molecule of) nucleic acid is present in each partition. In other examples, a cell sample may be partitioned such that no more than one cell is present in each partition.

Following the partitioning step, any of a number of different operations may be performed on the subdivided sample within the device. The partitions may include one or more capsules that contain one or more reagents (e.g., enzymes, unique identifiers (e.g., bar codes), antibodies, etc.). In some cases, the device, a companion device or a user provides a trigger that causes the microcapsules to release one or more of the reagents into the respective partition. The release of the reagent may enable contact of the reagent with the subdivided sample. For example, if the reagent is a unique identifier such as a barcode, the sample may be tagged with the unique identifier. The tagged sample may then be used in a downstream application such as a sequencing reaction.

A variety of different reactions and/operations may occur within a device disclosed herein, including but not limited to: sample partitioning, sample isolation, binding reactions, fragmentation (e.g., prior to partitioning or following partitioning), ligation reactions, and other enzymatic reactions. The device also may be useful for a variety of different molecular biology applications including, but not limited to, nucleic acid sequencing, protein sequencing, nucleic acid quantification, sequencing optimization, detecting gene expression, quantifying gene expression, and single-cell analysis of genomic or expressed markers. Moreover, the device has numerous medical applications. For example, it may be used for the identification, detection, diagnosis, treatment, staging of, or risk prediction of various genetic and non-genetic diseases and disorders including cancer.

II. Microcapsules

FIG. **1**A is a schematic of an exemplary microcapsule comprising an internal compartment **120** enveloped by a second layer **130**, which is encapsulated by a solid or semi-permeable shell or membrane **110**. In general, the shell separates the internal compartment(s) from their immediate environment (e.g., interior of a microwell). The internal compartments, e.g., **120**, **130**, may comprise materials such as reagents. As depicted in FIG. **1**A, the reagents **100** may be present in the internal compartment **120**. However, in some cases, the reagents are located in the enveloping layer **130** or in both compartments. Generally, the microcapsule may release the inner materials, or a portion thereof, following the introduction of a particular trigger. The trigger may cause disruption of the shell layer **110** and/or the internal enveloping layer **130**, thereby permitting contact of the internal compartment **100**, **120** with the outside environment, such as the cavity of a microwell.

The microcapsule may comprise several fluidic phases and may comprise an emulsion (e.g. water-in-oil emulsion, oil-in-water emulsion). A microcapsule may comprise an internal layer **120** that is immiscible with a second layer **130** enveloping the internal layer. For example, the internal layer **120** may comprise an aqueous fluid, while the enveloping layer **130** may be a non-aqueous fluid such as an oil. Conversely, the internal layer **120** may comprise a non-aqueous fluid (e.g., oil), and the enveloping layer **130** may comprise an aqueous fluid. In some cases, the microcapsule does not comprise an enveloping second layer. Often, the microcapsule is further encapsulated by a shell layer **110**, which may comprise a polymeric material. In some cases, a microcapsule may comprise a droplet.

Droplets and methods for droplet generation, for example, are described in U.S. Pat. No. RE41,780, which is incorporated herein by reference in its entirety for all purposes. The device also may contain a microfluidic element that enables the flow of a sample and/or microcapsules through the device and distribution of the sample and/or microcapsules within the partitions.

The microcapsule can comprise multiple compartments. The microcapsule may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 compartments. In other cases, the microcapsule comprises less than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 compartments. Similarly, each compartment, or a subset thereof, may also be subdivided into a plurality of additional compartments. In some cases, each compartment, or subset thereof, is subdivided into at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 compartments. In other cases, each compartment, or subset thereof, is further subdivided into less than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 compartments.

There are several possible distributions of reagent in the multiple compartments. For example, each compartment (or some percentage of the total number of compartments) may comprise the same reagent or the same combination or reagents. In some cases, each compartment (or some percentage of the total number of compartments) comprises different reagents or a different combination of reagents.

The compartments may be configured in a variety of ways. In some cases, the microcapsule may comprise multiple concentric compartments (repeating units of compartments that contain the preceding compartment), often separated by an immiscible layer. In such microcapsules, the reagents may be present in alternating compartments, in every third compartment, or in every fourth compartment.

In some cases, most of the compartments with a microcapsule are not concentric; instead, they exist as separate, self-contained entities within a microcapsule. FIG. **1**B depicts an example of a microcapsule that contains a plurality of smaller microcapsules **140**, each containing a reagent. Like many of the other microcapsules described herein, the microcapsule may be encapsulated by an outer shell, often comprising a polymer material **150**. The plurality of smaller microcapsules encapsulated within the larger microcapsule may be physically separated by an immiscible fluid **160**, thereby preventing mixing of reagents before application of a stimulus and release of reagents into solution. In some cases, the immiscible fluid is loaded with additional materials or reagents. In some cases, the plurality of smaller microcapsules are surrounded by a layer of immiscible fluid (e.g., **170**) which is further surrounded by a fluid **160** that is miscible with the inner fluid of the microcapsules. For example, the interior microcapsules **180** may comprise an aqueous interior enveloped by an immiscible (e.g., oil) layer, that is further surrounded by an aqueous layer **160**. The miscible compartments (e.g., **160** and **180**) may each contain reagents. They may contain the same reagents (or the same combination of reagents) or different reagents (or different combination of reagents). Alternatively, one or some of the miscible compartments may comprise no reagents.

The microcapsule may comprise a polymeric shell (see, e.g., FIGS. **1** and **2**) or multiple polymeric shells. For example, the microcapsule may comprise multiple polymeric shells layered on top of each other. In other cases, individual compartments within a microcapsule comprise a polymeric shell, or a subset of the compartments may comprise a polymeric shell. For example, all or some of the smaller compartments **140** in FIG. **1**B may comprise a polymeric shell that separates them from the fluidic interior **160**. The microcapsule may be designed so that a particular reagent is contained within a compartment that has a polymerized shell, while a different reagent is within a compartment that is simply enveloped by an immiscible liquid. For example, a reagent that is desired to be released upon a heat trigger may be contained within the compartments that have a heat-sensitive or heat-activatable polymerized shell, while reagents designed to be released upon a different trigger may be present in different types of compartments. In another example, paramagnetic particles may be incorporated into the capsule shell wall. A magnet or electric field may then be used to position the capsule to a desired location. In some cases, a magnetic field (e.g., high frequency alternating magnetic field) can be applied to such capsules; the incorporated paramagnetic particles may then transform the energy of the magnetic field into heat, thereby triggering rupture of the capsule.

The microcapsule component of a device of this disclosure may provide for the controlled and/or timed release of reagents for sample preparation of an analyte. Microcapsules may be used in particular for controlled release and transport of varying types of chemicals, ingredients, pharmaceuticals, fragrances etc . . . , including particularly

sensitive reagents such as enzymes and proteins (see, e.g., D. D. Lewis, "Biodegradable Polymers and Drug Delivery Systems", M. Chasin and R. Langer, editors (Marcel Decker, N.Y., 1990); J. P. McGee et al., J. Control. Release 34 (1995), 77).

Microcapsules may also provide a means for delivery of reagents in discrete and definable amounts. Microcapsules may be used to prevent premature mixing of reagents with the sample, by segregating the reagents from the sample. Microcapsules also may ease handling of—and limit contacts with—particularly sensitive reagents such as enzymes, nucleic acids and other chemicals used in sample preparation.

A. Preparation of Microcapsules

Microcapsules of a device of this disclosure may be prepared by numerous methods and processes. Preparative techniques may include pan coating, spray drying, centrifugal extrusion, emulsion-based methods, and/or microfluidic techniques. Typically, a method for preparation is chosen based on the desired characteristics of the microcapsule. For example, shell wall thickness, permeability, chemical composition of the shell wall, mechanical integrity of the shell wall and capsule size may be taken into consideration when choosing a method. Methods of preparation may also be selected based on the ability to incorporate specific materials within the capsule such as whether the core materials (e.g., fluids, reagents, etc.) are aqueous, organic or inorganic. Additionally, preparation methods can affect the shape and size of the microcapsule. For example a capsule's shape, (e.g., spherical, ellipsoidal, etc.), may depend on the shape of the droplet in the precursor liquid which may be determined by the viscosity and surface tension of the core liquid, direction of flow of the emulsion, the choice of surfactants used in droplet stabilization, as well as physical confinement such as preparations made in a microchannel or capillary of a particular size (e.g., a size requiring distortion of the microcapsule in order for the microcapsule to fit within the microchannel or capillary.

Microcapsules may be prepared through emulsification polymerization, a process in which monomer units at an aqueous/organic interface in an emulsion polymerize to form a shell. Reagents are mixed with the aqueous phase of the biphasic mixture. Vigorous shaking, or sonication of the mixture, creates droplets containing reagents, which are encased by a polymeric shell.

In some cases, microcapsules may be prepared through layer-by-layer assembly, a process in which negatively and positively charged polyelectrolytes are deposited onto particles such as metal oxide cores. Electrostatic interactions between polyelectrolytes create a polymeric shell around the core. The core can be subsequently removed via addition of acid, resulting in a semi-permeable hollow sphere which can be loaded with various reagents.

In still further cases, microcapsules may be prepared through coacervation, a process in which two oppositely charged polymers in aqueous solution become entangled to form a neutralized polymer shell wall. One polymer may be contained within an oil phase, while the other, of opposite charge is contained in an aqueous phase. This aqueous phase may contain reagents to be encapsulated. The attraction of one polymer for another can result in the formation of coascervates. In some embodiments, gelatin and gum Arabic are components of this preparative method.

Microcapsules also may be prepared through internal phase separation, a process in which a polymer is dissolved in a solvent mixture containing volatile and nonvolatile solvents. Droplets of the resultant solution are suspended in an aqueous layer, which is stabilized by continual agitation and the use of surfactants. This phase may contain reagents to be encapsulated. When the volatile solvent evaporates, the polymers coalesce to form a shell wall. In some cases, polymers such as polystyrene, poly(methyl methacrylate) and poly(tetrahydrofuran) are used to form shell walls.

Microcapsules also may be prepared through flow focusing methods, a process in which a microcapillary device is used to generate double emulsions containing a single internal droplet encased in a middle fluid which is then dispersed to an outer fluid. The inner droplet may contain reagents to be encapsulated. The middle fluid becomes the shell wall, which can be formed via cross-linking reactions.

B. Microcapsule Composition

Microcapsules may comprise a variety of materials with a wide range of chemical characteristics. Generally, the microcapsules comprise materials with the ability to form microcapsules of a desired shape and size and that are compatible with the reagents to be stored in the microcapsules.

Microcapsules may comprise a wide range of different polymers including but not limited to: polymers, heat sensitive polymers, photosensitive polymers, magnetic polymers, pH sensitive polymers, salt-sensitive polymers, chemically sensitive polymers, polyelectrolytes, polysaccharides, peptides, proteins, and/or plastics. Polymers may include but are not limited to materials such as poly(N-isopropylacrylamide) (PNIPAAm), poly(styrene sulfonate) (PSS), poly(allyl amine) (PAAm), poly(acrylic acid) (PAA), poly(ethylene imine) (PEI), poly(diallyldimethyl-ammonium chloride) (PDADMAC), poly(pyrolle) (PPy), poly (vinylpyrrolidone) (PVPON), poly(vinyl pyridine) (PVP), poly(methacrylic acid) (PMAA), poly(methyl methacrylate) (PMMA), polystyrene (PS), poly(tetrahydrofuran) (PTHF), poly(phthaladehyde) (PTHF), poly(hexyl viologen) (PHV), poly(L-lysine) (PLL), poly(L-arginine) (PARG), poly(lactic-co-glycolic acid) (PLGA).

Often, materials for the microcapsules, particularly the shells of microcapsules, may enable the microcapsule to be disrupted with an applied stimulus. For example, a microcapsule may be prepared from heat sensitive polymers and/or may comprise one or more shells comprising such heat-sensitive polymers. The heat-sensitive polymer may be stable under conditions used for storage or loading. Upon exposure to heat, the heat-sensitive polymer components may undergo depolymerization, resulting in disruption to the integrity of the shell and release of the inner materials of the microcapsule (and/or of the inner microcapsules) to the outside environment (e.g., the interior of a microwell). Exemplary heat-sensitive polymers may include, but are not limited to NIPAAm or PNIPAM hydrogel. The microcapsules may also comprise one or more types of oil. Exemplary oils include but are not limited to hydrocarbon oils, fluorinated oils, fluorocarbon oils, silicone oils, mineral oils, vegetable oils, and any other suitable oil.

The microcapsules may also comprise a surfactant, such as an emulsifying surfactant. Exemplary surfactants include, but are not limited to, cationic surfactants, non-ionic surfactants, anionic surfactants, hydrocarbon surfactants or fluorosurfactants. The surfactant may increase the stability of one or more components of the microcapsule, such as an inner compartment that comprises an oil.

Additionally, the microcapsules may comprise an inner material that is miscible with materials external to the capsule. For example, the inner material may be an aqueous fluid and the sample within the microwell may also be in an aqueous fluid. In other examples, the microcapsule may

comprise powders or nanoparticles that are miscible with an aqueous fluid. For example, the microcapsule may comprise such powders or nanoparticles in an inner compartment. Upon disruption of the microcapsule, such powders or nanoparticles are released into the external environment (e.g., interior of microwell) and may mix with an aqueous fluid (e.g., an aqueous sample fluid).

Additionally, the microcapsule may comprise a material that is immiscible with the surrounding environment (e.g., interior of microwell, sample fluid). In such cases, when the inner emulsion is released to the surrounding environment, the phase separation between the inner and outer components may promote mixing, such as mixing of the inner components with the surrounding fluid. In some cases, when a microcapsule is triggered to release its contents, a pressure or force is also released that promotes mixing of internal and external components.

The microcapsules may also comprise a polymer within the interior of the capsule. In some instances this polymer may be a porous polymer bead that may entrap reagents or combinations of reagents. In other instances, this polymer may be a bead that has been previously swollen to create a gel. Examples of polymer based gels that may be used as inner emulsions of capsules may include, but are not limited to sodium alginate gel, or poly acrylamide gel swelled with oligonucleotide bar codes or the like.

In some cases, a microcapsule may be a gel bead comprising any of the polymer based gels described herein. Gel bead microcapsules may be generated, for example, by encapsulating one or more polymeric precursors into droplets. Upon exposure of the polymeric precursors to an accelerator (e.g., tetramethylethylenediamine (TEMED)), a gel bead may be generated.

Analytes and/or reagents, such as oligonucleotide barcodes, for example, may be coupled/immobilized to the interior surface of a gel bead (e.g., the interior accessible via diffusion of an oligonucleotide barcode and/or materials used to generate an oligonucleotide barcode) and/or the outer surface of a gel bead or any other microcapsule described herein. Coupling/immobilization may be via any form of chemical bonding (e.g., covalent bond, ionic bond) or physical phenomena (e.g., Van der Waals forces, dipole-dipole interactions, etc.). In some cases, coupling/immobilization of a reagent to a gel bead or any other microcapsule described herein may be reversible, such as, for example, via a labile moiety (e.g., via a chemical cross-linker, including chemical cross-linkers described herein). Upon application of a stimulus, the labile moiety may be cleaved and the immobilized reagent set free. In some cases, the labile moiety is a disulfide bond. For example, in the case where an oligonucleotide barcode is immobilized to a gel bead via a disulfide bond, exposure of the disulfide bond to a reducing agent can cleave the disulfide bond and free the oligonucleotide barcode from the bead. The labile moiety may be included as part of a gel bead or microcapsule, as part of a chemical linker that links a reagent or analyte to a gel bead or microcapsule, and/or as part of a reagent or analyte.

A gel bead or any other type of microcapsule described herein may contain varied numbers of reagents. The density of a reagent per microcapsule may vary depending on the particular microcapsule utilized and the particular reagent. For example, a microcapsule or gel bead may comprise at least about 1; 10; 100; 1,000; 10,000; 100,000; 1,000,000; 5,000,000; 10,000,000, 50,000,000; 100,000,000; 500,000,000; or 1,000,000,000 oligonucleotide barcodes per microcapsule or gel bead. A gel bead may comprise identical oligonucleotide barcodes or may comprise differing oligonucleotide barcodes.

In other example, the microcapsule may comprise one or more materials that create a net neutral, negative or positive charge on the outer shell wall of the capsule. In some instances, the charge of a capsule may aid in preventing or promoting aggregation or clustering of particles, or adherence or repulsion to parts of the device.

In addition, the microcapsule may comprise one or more materials that cause the outer shell wall of the capsule to be hydrophilic or hydrophobic. A hydrophilic material that may be used for capsule shell walls may be poly(N-isopropylacrylamide). A hydrophobic material that may be used for capsule shell walls may be polystyrene. In certain instances, a hydrophilic shell wall may aid in wicking of the capsule into wells comprising aqueous fluid.

C. Microcapsule Size and Shape

A microcapsule may be any of a number of sizes or shapes. In some cases, the shape of the microcapsule may be spherical, ellipsoidal, cylindrical, hexagonal or any other symmetrical or non-symmetrical shape. Any cross-section of the microcapsule may also be of any appropriate shape, include but not limited to: circular, oblong, square, rectangular, hexagonal, or other symmetrical or non-symmetrical shape. In some cases, the microcapsule may be of a specific shape that complements an opening (e.g., surface of a microwell) of the device. For example, the microcapsule may be spherical and the opening of a microwell of the device may be circular.

The microcapsules may be of uniform size (e.g., all of the microcapsules are the same size) or heterogeneous size (e.g., some of the microcapsules are of different sizes). A dimension (e.g., diameter, cross-section, side, etc.) of a microcapsule may be at least about 0.001 μm, 0.01 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm or 1 nm. In some cases, the microcapsule comprises a microwell that is at most about 0.001 μm, 0.01 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm or 1 nm.

In some cases, microcapsules are of a size and/or shape so as to allow a limited number of microcapsules to be deposited in individual partitions (e.g., microwells, droplets) of the microcapsule array. Microcapsules may have a specific size and/or shape such that exactly or no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 capsules fit into an individual microwell; in some cases, on average 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 capsules fit into an individual microwell. In still further cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 100, 500, or 1000 capsules fit into an individual microwell.

D. Reagents and Reagent Loading

The devices provided herein may comprise free reagents and/or reagents encapsulated into microcapsules. The reagents may be a variety of molecules, chemicals, particles, and elements suitable for sample preparation reactions of an analyte. For example, a microcapsule used in a sample preparation reaction for DNA sequencing of a target may comprise one or more of the following reagents: enzymes, restriction enzymes (e.g., multiple cutters), ligase, polymerase (e.g., polymerases that do and do not recognize dUTPs and/or uracil), fluorophores, oligonucleotide barcodes, buffers, deoxynucleotide triphosphates (dNTPs) (e.g. deoxyadenosine triphosphate (dATP), deoxycitidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP), deoxyuridine triphosphate (dUTP)), deoxynucleotide triphosphates (ddNTPs) and the

like. In another example, a microcapsule used in a sample preparation reaction for single cell analysis may comprise reagents such as one or more of the following reagents: lysis buffer, detergent, fluorophores, oligonucleotide barcodes, ligase, proteases, heat activatable proteases, protease or nuclease inhibitors, buffer, enzymes, antibodies, nanoparticles, and the like.

Exemplary reagents include, but are not limited to: buffers, acidic solution, basic solution, temperature-sensitive enzymes, pH-sensitive enzymes, light-sensitive enzymes, metals, metal ions, magnesium chloride, sodium chloride, manganese, aqueous buffer, mild buffer, ionic buffer, inhibitor, enzyme, protein, nucleic acid, antibodies, saccharides, lipid, oil, salt, ion, detergents, ionic detergents, non-ionic detergents, oligonucleotides, nucleotides, dNTPs, ddNTPs, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), peptide nucleic acids, circular DNA (cDNA), double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), plasmid DNA, cosmid DNA, chromosomal DNA, genomic DNA (gDNA), viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), nRNA, short-interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajul body specific RNA, (scaRNA), microRNA, double-stranded RNA (dsRNA), ribozyme, riboswitch and viral RNA, polymerase (e.g., polymerases that do and do not recognize dUTPs and/or uracil), ligase, restriction enzymes, proteases, nucleases, protease inhibitors, nuclease inhibitors, chelating agents, reducing agents (e.g., dithiotheritol (DTT), 2-tris(2-carboxyethyl)phosphine (TCEP)), oxidizing agents, fluorophores, probes, chromophores, dyes, organics, emulsifiers, surfactants, stabilizers, polymers, water, small molecules, pharmaceuticals, radioactive molecules, preservatives, antibiotics, aptamers, and pharmaceutical drug compounds.

In some cases, a microcapsule comprises a set of reagents that have a similar attribute (e.g., a set of enzymes, a set of minerals, a set of oligonucleotides, a mixture of different bar-codes, a mixture of identical bar-codes). In other cases, a microcapsule comprises a heterogeneous mixture of reagents. In some cases, the heterogeneous mixture of reagents comprises all components necessary to perform a reaction. In some cases, such mixture comprises all components necessary to perform a reaction, except for 1, 2, 3, 4, 5, or more components necessary to perform a reaction. In some cases, such additional components are contained within a different microcapsule or within a solution within a partition (e.g., microwell) of the device.

Reagents may be pre-loaded into the device (e.g., prior to introduction of analyte) or post-loaded into the device. They may be loaded directly into the device; or, in some cases, the reagents are encapsulated into a microcapsule that is loaded into the device. In some cases, only microcapsules comprising reagents are introduced. In other cases, both free reagents and reagents encapsulated in microcapsules are loaded into the device, either sequentially or concurrently. In some cases, reagents are introduced to the device either before or after a particular step. For example, a lysis buffer reagent may be introduced to the device following partitioning of a cellular sample into multiple partitions (e.g., microwells, droplets) within the device. In some cases, reagents and/or microcapsules comprising reagents are introduced sequentially such that different reactions or operations occur at different steps. The reagents (or microcapsules) may be also be loaded at steps interspersed with a reaction or operation step. For example, microcapsules comprising reagents for fragmenting molecules (e.g., nucleic acids) may be loaded into the device, followed by a fragmentation step, which may be followed by loading of microcapsules comprising reagents for ligating bar-codes (or other unique identifiers, e.g., antibodies) and subsequent ligation of the bar-codes to the fragmented molecules. Additional methods of loading reagents are described further herein in other sections.

E. Molecular 'Barcodes'

It may be desirable to retain the option of identifying and tracking individual molecules or analytes after or during sample preparation. In some cases, one or more unique molecular identifiers, sometimes known in the art as a 'molecular barcodes,' are used as sample preparation reagents. These molecules may comprise a variety of different forms such as oligonucleotide bar codes, antibodies or antibody fragments, fluorophores, nanoparticles, and other elements or combinations thereof. Depending upon the specific application, molecular barcodes may reversibly or irreversibly bind to the target analyte and allow for identification and/or quantification of individual analytes after recovery from a device after sample preparation.

A device of this disclosure may be applicable to nucleic acid sequencing, protein detection, single molecule analysis and other methods that require a) precise measurement of the presence and amount of a specific analyte b) multiplex reactions in which multiple analytes are pooled for analysis. A device of this disclosure may utilize the microwells of the microwell array or other type of partition (e.g., droplets) to physically partition target analytes. This physical partitioning allows for individual analytes to acquire one or more molecular barcodes. After sample preparation, individual analytes may be pooled or combined and extracted from a device for multiplex analysis. For most applications, multiplex analysis substantially decreases the cost of analysis as well as increases through-put of the process, such as in the case of the nucleic acid sequencing. Molecular barcodes may allow for the identification and quantification of individual molecules even after pooling of a plurality of analytes. For example, with respect to nucleic acid sequencing, molecular barcodes may permit the sequencing of individual nucleic acids, even after the pooling of a plurality of different nucleic acids.

Oligonucleotide barcodes, in some cases, may be particularly useful in nucleic acid sequencing. In general, an oligonucleotide barcode may comprise a unique sequence (e.g., a barcode sequence) that gives the oligonucleotide barcode its identifying functionality. The unique sequence may be random or non-random. Attachment of the barcode sequence to a nucleic acid of interest may associate the barcode sequence with the nucleic acid of interest. The barcode may then be used to identify the nucleic acid of interest during sequencing, even when other nucleic acids of interest (e.g., comprising different barcodes) are present. In cases where a nucleic acid of interest is fragmented prior to sequencing, an attached barcode may be used to identify fragments as belonging to the nucleic acid of interest during sequencing.

An oligonucleotide barcode may consist solely of a unique barcode sequence or may be included as part of an oligonucleotide of longer sequence length. Such an oligonucleotide may be an adaptor required for a particular sequencing chemistry and/or method. For example, such adaptors may include, in addition to an oligonucleotide barcode, immobilization sequence regions necessary to immobilize (e.g., via hybridization) the adaptor to a solid surface (e.g., solid surfaces in a sequencer flow cell channel); sequence regions required for the binding of sequenc-

ing primers; and/or a random sequence (e.g., a random N-mer) that may be useful, for example, in random amplification schemes. An adaptor can be attached to a nucleic acid to be sequenced, for example, by amplification, ligation, or any other method described herein.

Moreover, an oligonucleotide barcode, and/or a larger oligonucleotide comprising an oligonucleotide barcode may comprise natural nucleic acid bases and/or may comprise non-natural bases. For example, in cases where an oligonucleotide barcode or a larger oligonucleotide comprising an oligonucleotide barcode is DNA, the oligonucleotide may comprise the natural DNA bases adenine, guanine, cytosine, and thymine and/or may comprise non-natural bases such as uracil.

F. Microcapsule-Preparation for Microwell Loading

Following preparation, reagent loaded microcapsules may be loaded into a device using a variety of methods. Microcapsules, in some instances, may be loaded as 'dry capsules.' After preparation, capsules may be separated from a liquid phase using various techniques, including but not limited to differential centrifugation, evaporation of the liquid phase, chromatography, filtration and the like. 'Dry capsules' may be collected as a powder or particulate matter and then deposited into microwells of the microwell array. Loading 'dry capsules' may be a preferred method in instances in which loading of 'wet capsules,' leads to inefficiencies of loading such as empty wells and poor distribution of microcapsules across the microwell array.

Reagent-loaded microcapsules may also be loaded into a device when the microcapsules are within a liquid phase, and thereby loaded as 'wet capsules.' In some instances, microcapsules may be suspended in a volatile oil such that the oil can be removed or evaporated, leaving only the dry capsule in the well. Loading 'wet capsules' may be a preferred method in some instances in which loading of dry capsules leads to inefficiencies of loading, such as microcapsule clustering, aggregation and poor distribution of microcapsules across the microwell array. Additional methods of loading reagents and microcapsules are described in other sections of this disclosure.

The microcapsules also may have a particular density. In some cases, the microcapsules are less dense than an aqueous fluid (e.g., water); in some cases, the microcapsules are denser than an aqueous fluid (e.g., water). In some cases, the microcapsules are less dense than a non-aqueous fluid (e.g., oil); in some cases, the microcapsules are denser than a non-aqueous fluid (e.g., oil). Microcapsules may comprise a density at least about 0.05 g/cm^3, 0.1 cm^3, 0.2 g/cm^3, 0.3 g/cm^3, 0.4 g/cm^3, 0.5 g/cm^3, 0.6 g/cm^3, 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. In other cases, microcapsule densities may be at most about 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. Such densities can reflect the density of the microcapsule in any particular fluid (e.g., aqueous, water, oil, etc.)

III. Microwell Array

A. Structure/Features

A device of this disclosure may be a microwell array comprising a solid plate containing a plurality of holes, cavities or microwells in which microcapsules and/or analytes are deposited. Generally, a fluidic sample (or analyte) is introduced into the device (e.g., through an inlet) and then travels through a flow channel which distributes the sample into multiple microwells. In some cases, additional fluid is introduced into the device as well. The microwells may comprise microcapsules when the sample is introduced; or, in some cases, the microcapsules are introduced into the microwells following introduction of the sample.

FIG. **2**A depicts a prototype microwell array; a sideview is depicted in FIG. **2**B. The microwell array may include a plate **220** that can be made of any suitable material commonly used in a chemical laboratory, including fused silica, soda lima glass, borosilicate glass, PMMA, sapphire, silicon, germanium, cyclic olefin copolymer and cyclic polymer, polyethylenes, polypropylenes, polyacrylates, polycarbonates, plastics, Topas, and other suitable substrates known in the art. The plate **220** may initially be a flat solid plate comprising a regular pattern of microwells **270**. The microwells may be formed by drilling or chemical dissolution or any other suitable method of machining; however, plates with a desired hole pattern are preferably molded, e.g. by injection-molding, embossing, or using a suitable polymer, such as cyclic olefin copolymer.

The microwell array may comprise an inlet (**200** and **240**) and/or an outlet (**210** and **260**); in some cases, the microwell array comprises multiple inlets and/or outlets. A sample (or analyte) or microcapsules may be introduced to the device via the inlet. Solutions containing analytes, reagents and/or microcapsules may be manually applied to the inlet port **200** and **240** (or to a conduit attached to the inlet port) via a pipette. In some cases, a liquid handling device is used to introduce analytes, reagents, and/or microcapsules to the device. Exemplary liquid handling devices may rely on a pipetting robot, capillary action, or dipping into a fluid. In some cases, the inlet port is connected to a reservoir comprising microcapsules or analytes. The inlet port may be attached to a flow channel **250** that permits distribution of the analyte, sample, or microcapsules to the microwells in the device. In some cases, the inlet port may be used to introduce to the device a fluid (e.g., oil, aqueous) that does not contain microcapsules or analyte, such as a carrier fluid. The carrier fluid may be introduced via the inlet port before, during, or following the introduction of analyte and/or microcapsules. In cases where the device has multiple inlets, the same sample may be introduced via the multiple inlets, or each inlet may convey a different sample. In some cases, one inlet may convey a sample or analyte to the microwells, while a different inlet conveys free reagents and/or reagents encapsulated in microcapsules to the device. The device may have at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 inlets and/or outlets.

In some cases, solutions containing microcapsules and/or analytes may be pulled through the device via a vacuum manifold attached to the outlet port **210** and **260**. Such manifold may apply a negative pressure to the device. In other cases, a positive pressure is used to move sample, analytes, and/or microcapsules through the device. The area, length, and width of surfaces of 230 according to this disclosure may be varied according to the requirements of the assay to be performed. Considerations may include, for example, ease of handling, limitations of the material(s) of which the surface is formed, requirements of detection or processing systems, requirements of deposition systems

(e.g. microfluidic systems), and the like. The thickness may comprise a thickness of at least about 0.001 mm, 0.005 mm, 0.01 mm, 0.05 mm, 0.1 mm, 0.2 mm, 0.3 mm, 0.4 mm, 0.5 mm, 0.6 mm, 0.7 mm, 0.8 mm, 0.9 mm, 1.0 mm, 2.0 mm, 3.0 mm, 4.0 mm, 5.0 mm, 6.0 mm, 7.0 mm, 8.0 mm, 9.0 mm, 10.0 mm, 11 mm, 12 mm, 13 mm, 14 mm, or 15 mm. In other cases, microcapsule thickness may be at most 0.001 mm, 0.005 mm, 0.01 mm, 0.05 mm, 0.1 mm, 0.2 mm, 0.3 mm, 0.4 mm, 0.5 mm, 0.6 mm, 0.7 mm, 0.8 mm, 0.9 mm, 1.0 mm, 2.0 mm, 3.0 mm, 4.0 mm, 5.0 mm, 6.0 mm, 7.0 mm, 8.0 mm, 9.0 mm, 10.0 mm, 11 mm, 12 mm, 13 mm, 14 mm, or 15 mm.

The microwells **270** can be any shape and size suitable for the assay performed. The cross-section of the microwells may have a cross-sectional dimension that is circular, rectangular, square, hexagonal, or other symmetric or non-symmetric shape. In some cases, the shape of the microwell may be cylindrical, cubic, conical, frustoconical, hexagonal or other symmetric or non-symmetric shape. The diameter of the microwells **270** may be determined by the size of the wells desired and the available surface area of the plate itself. Exemplary microwells comprise diameters of at least 0.01 μm, 0.1 μm, 0.2 μm, 0.3 μm, 0.4 μm, 0.5 μm, 1 μm, 10 μm, 25 μm, 50 μm, 75 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, 1.0 mm. In other cases, microwell diameters may comprise at most 0.01 μm, 0.1 μm, 0.2 μm, 0.3 μm, 0.4 μm, 0.5 μm, 1 μm, 10 μm, 25 μm, 50 μm, 75 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm or 1.0 mm.

The capacity (or volume) of each well can be a measure of the height of the well (the thickness of the plate) and the effective diameter of each well. The capacity of an individual well may be selected from a wide range of volumes. In some cases, the device may comprise a well (or microwell) with a capacity of at least 0.001 fL, 0.01 fL, 0.1 fL, 0.5 fL, 1 fL, 5 fL, 10 fL, 50 fL, 100 fL, 200 fL, 300 fL, 400 fL, 500 fL, 600 fL, 700 fL, 800 fL, 900 fL, 1 pL, 5 pL, 10 pL, 50 pL, 100 pL, 200 pL, 300 pL, 400 pL, 500 pL, 600 pL, 700 pL, 800 pL, 900 pL, 1 nL, 5 nL, 10 nL, 50 nL, 100 nL, 200 nL, 300 nL, 400 nL, 500 nL, 1 uL, 50 uL, or 100 uL. In other cases, the microcapsule comprises a microwell that is less than 0.001 fL, 0.01 fL, 0.1 fL, 0.5 L, 5 fL, 10 fL, 50 fL, 100 fL, 200 fL, 300 fL, 400 fL, 500 fL, 600 fL, 700 fL, 800 fL, 900 fL, 1 pL, 5 pL, 10 pL, 50 pL, 100 pL, 200 pL, 300 pL, 400 pL, 500 pL, 600 pL, 700 pL, 800 pL, 900 pL, 1 nL, 5 nL, 10 nL, 50 nL, 100 nL, 200 nL, 300 nL, 400 nL, 500 nL, 1 uL, 50 uL, or 100 uL.

There may be variability in the volume of fluid in different microwells in the array. More specifically, the volume of different microwells may vary by at least (or at most) plus or minus 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, or 1000% across a set of microwells. For example, a microwell may comprise a volume of fluid that is at most 80% of the fluid volume within a second microwell.

Based on the dimension of individual microwells and the size of the plate, the microwell array may comprise a range of well densities. In some examples, a plurality of microwells may have a density of at least about 2,500 wells/cm^2, at least about 1,000 wells/cm^2. in some cases, the plurality of wells may have a density of at least 10 wells/cm^2. In other cases, the well density may comprise at least 10 wells/cm^2, 50 wells/cm^2, 100 wells/cm^2, 500 wells/cm^2, 1000 wells/cm^2, 5000 wells/cm^2, 10000 wells/cm^2, 50000 wells/cm^2, or 100000 wells/cm^2. In other cases, the well density may be less than 100000 wells/cm^2, 10000 wells/cm^2, 5000 wells/cm^2, 1000 wells/cm^2, 500 wells/cm^2, or 100 wells/cm^2.

In some cases, the interior surface of the microwells comprises a hydrophilic material that preferably accommodates an aqueous sample; in some cases, the region between the microwells is composed of a hydrophobic material that may preferentially attract a hydrophobic sealing fluid described herein.

Multiple microwell arrays, e.g., FIG. **2**B may be arranged within a single device. FIG. **3**, **300**. For example, discrete microwell array slides may be arrayed in parallel on a plate holder. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 25, 50 or 100 microwell arrays are arrayed in parallel. In other cases, at most 100, 50, 25, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 devices are arrayed in parallel. The microwell arrays within a common device may be manipulated simultaneously or sequentially. For example, arrayed devices may be loaded with samples or capsules simultaneously or sequentially.

B. Microwell Array Fluids

The microwell array may comprise any of a number of different fluids including aqueous, non-aqueous, oils, and organic solvents, such as alcohols. In some cases, the fluid is used to carry a component, e.g., reagent, microcapsule, or analyte, to a target location such as microwells, output port, etc. In other cases, the fluid is used to flush the system. In still other cases, the fluid may be used to seal the microwells.

Any fluid or buffer that is physiologically compatible with the analytes (e.g., cells, molecules) or reagents used in the device may be used. In some cases, the fluid is aqueous (buffered or not buffered). For example, a sample comprising a population of cells suspended in a buffered aqueous solution may be introduced into the microwell array, allowed to flow through the device, and distributed to the microwells. In other cases, the fluid passing through the device is nonaqueous (e.g., oil). Exemplary non-aqueous fluids include but are not limited to: oils, non-polar solvent, hydrocarbon oil, decane (e.g., tetradecane or hexadecane), fluorocarbon oil, fluorinated oil, silicone oil, mineral oil, or other oil.

Often, the microcapsules are suspended in a fluid that is compatible with the components of the shell of the microcapsule. Fluids including but not limited to water, alcohols, hydrocarbon oils or fluorocarbon oils are particularly useful fluids for suspending and flowing microcapsules through the microarray device.

C. Further Partitioning and Sealing

After the analyte, free reagents, and/or microcapsules are loaded into the device and distributed to the microwells, a sealing fluid may be used to further partition or isolate them within the microwells. The sealing fluid may also be used to seal the individual wells. The sealing fluid may be introduced through the same inlet port that was used to introduce the analyte, reagents and/or microcapsules. But in some cases, the sealing fluid is introduced to the device by a separate inlet port, or through multiple separate inlet ports.

Often, the sealing fluid is a non-aqueous fluid (e.g., oil). When the sealing fluid flows through the microwell array device, it may displace excess aqueous solution (e.g., solution comprising analytes, free reagents and/or microcapsules) from individual microwells, thereby potentially removing aqueous bridges between adjacent microwells. The wells themselves, as described herein, may comprise a hydrophilic material that enables wicking of the aqueous fluids (e.g., sample fluid, microcapsule fluid) into individual wells. In some cases, regions external to the wells comprise hydrophobic material, again to encourage the positioning of the aqueous fluid into the interior of the microwells.

The sealing fluid may either remain in the device or be removed. The sealing fluid may be removed, e.g., by flowing

through the outlet port. In other cases, the sealing oil may comprise a volatile oil that can be removed by the application of heat. Once the sealing fluid is removed, analytes, free reagents and/or microcapsules may be physically partitioned from one another in the microwells.

A fluid may be selected such that its density is equal to, greater than or less than the density of the microcapsules. For example, the microcapsules may be denser than the sealing oil and/or aqueous fluid of the sample and reagents, thereby enabling the microcapsules to remain in the microwells as the sealing oil flows through the device. In another example, the capsules may be less dense than the aqueous fluid of the sample or the fluid that the microcapsules are suspended in, as described herein, thereby facilitating movement and distribution of the capsules across the plurality of microwells in a device.

In the case of microcapsules comprising paramagnetic material, a magnetic field may be used to load or direct the capsules into the microwells. A magnetic field may also be used to retain such microcapsules within the wells while the wells are being filled with sample, reagent, and/or sealing fluids. The magnetic field may also be used to remove capsule shells from the wells, particularly following rupture of the capsules.

In some cases, the sealing fluid may remain in the microwells when operations or reactions are conducted therein. The presence of the sealing fluid may act to further partition, isolate, or seal the individual microwells. In other cases, the sealing fluid may act as a carrier for the microcapsules. For example, sealing fluid comprising microcapsules may be introduced to the device to facilitate distribution of the microcapsules to the individual microwells. For such applications, the sealing fluid may be denser than the microcapsules in order to encourage more even distribution of the microcapsules to the microwells. Upon application of a stimulus, the microcapsules within the sealing fluid may release reagents to the microwell. In some cases, the sealing fluid may comprise a chemical or other agent capable of traveling from the sealing fluid to a well (e.g., by leaching or other mechanism) and triggering capsule rupture, where the capsule is present within the microwell or within the sealing fluid.

Methods other than those involving sealing fluids may also be used to seal the microwells following the loading of the analyte, free reagents, and/or microcapsules. For example, the microwells may be sealed with a laminate, tape, plastic cover, oils, waxes, or other suitable material to create an enclosed reaction chamber. The sealants described herein may protect the contents of the microwells from evaporation or other unintended consequences of the reactions or operations. Prevention of evaporation may be particularly necessary when heat is applied to the device, e.g., when heat is applied to stimulate microcapsule release.

In some cases, the laminate seal may also allow recovery of contents from individual wells. In this case, a single well of interest may be unsealed (e.g., by removal of the laminate seal) at a given time in order to enable further analysis of an analyte such as by MALDI mass spectrometry. Such applications may be useful in a number of settings, including high-throughput drug screening.

III. Loading Step(s)

As described herein, analytes, free reagents, and/or microcapsules may be loaded into the present device in any appropriate manner or order. The loading may be random or non-random. In some cases, a precise number of analytes and/or microcapsules are loaded into each individual microwell. In some cases, a precise number of analytes and/or microcapsules are loaded into a particular subset of microwells in the plate. In still other cases, an average number of analytes and/or micrcocapsules are loaded into each individual microwell. Furthermore, as described herein, in some cases, "dry" microcapsules are loaded into the device, while in other cases "wet" microcapsules are loaded into the device. In some cases, a combination of "dry" and "wet" microcapsules and/or reagents are loaded into the device, either simultaneously or sequentially.

As mentioned herein, the loading of the device may occur in any order and may occur in multiple stages. In some cases, the microcapsules are pre-loaded into the device, prior to the loading of the analyte. In other cases, the microcapsules and analyte are loaded concurrently. In still other cases, the analytes are loaded before the microcapsules are loaded.

The microcapsules and/or analytes may be loaded in multiple stages or multiple times. For example, microcapsules may be loaded into the device both prior to and after analytes are loaded into the device. The microcapsules that are pre-loaded (e.g., loaded prior to the analyte introduction) may comprise the same reagents as the microcapsules loaded after the analyte introduction. In other cases, the pre-loaded microcapsules contain reagents that are different from the reagents within the microcapsules loaded after analyte introduction. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 different sets of microcapsules are loaded onto the device. In some cases, the different sets of microcapsules are loaded sequentially; or, different sets of microcapsules may also be loaded simultaneously. Similarly, multiple sets of analytes can be loaded into the device. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 different sets of analytes are loaded onto the device. In some cases, the different sets of analytes are loaded sequentially; or, different sets of analytes may also be loaded simultaneously.

This disclosure provides devices comprising certain numbers of microcapsules and/or analytes loaded per well. In some cases, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, or 100 microcapsules and/or analytes are loaded into each individual microwell. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, or 100 microcapsules and/or analytes are loaded into each individual microwell. In some cases, on average, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, or 100 microcapsules and/or analytes are loaded into each individual microwell. In other cases, on average, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, or 100 microcapsules and/or analytes are loaded into each individual microwell. In some cases, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, or 100 microcapsules and/or analytes are loaded into each individual microwell.

Analytes and/or microcapsules may be applied in a quantity that allows a desired number of analytes to be deposited into an individual microwell. For example, terminal dilution of analytes, such as cells, may achieve the loading of one cell per one microwell or any desired number of analytes per microwell. In some cases, a Poisson distribution is used to direct or predict the final concentration of analytes or microcapsules per well.

The microcapsules may be loaded into the microarray device in a particular pattern. For example, certain sections of the device may comprise microcapsules containing a particular reagent (e.g., unique bar-code, enzyme, antibody, antibody subclass, etc.), while other sections of the device may comprise microcapsules containing a different reagent (e.g., a different bar-code, different enzyme, different antibody different antibody subclass, etc.). In some cases, the

microcapsules in one section of the array may contain control reagents. For example, they may contain positive controls that include a control analyte and necessary materials for a reaction. Or, in some cases, the microcapsules contain negative control reagents such as deactivated enzyme, or a synthetic oligonucleotide sequence that is resistant to fragmentation. In some cases, negative control reagents may control for the specificity of the sample preparation reaction etc. In other cases, the negative control microcapsules may comprise the same reagents present in other microcapsules except that the negative control microcapsule may lack a certain reagent (e.g., lysis buffer, polymerase, etc.).

The analytes/sample also may be loaded into the microarray device in a particular pattern. For example, certain sections of the device may comprise particular analytes, such as control analytes or analytes deriving from a particular source. This may be used in combination with specific loading of bar codes into known well locations. This feature may allow mapping of specific locations on the array to sequence data, thereby reducing the number of bar codes to be used for labeling reactions.

In cases where a partition is a droplet, an analyte and reagents may be combined within the droplet with the aid of a microfluidic device. For example, a droplet may be generated that comprises a gel bead (e.g., comprising an oligonucleotide barcode) a nucleic acid analyte, and any other desired reagents. The gel bead, nucleic acid analyte, and reagents in an aqueous phase may be combined at a junction of two or more channels of a microfluidic device. At a second junction of two or more channels of the microfluidic device, a droplet comprising the resulting mixture may be generated by contacting the aqueous mixture of reagents, gel bead, and nucleic acid analyte with an oil continuous phase.

IV. Microcapsule Stimuli

Various different stimuli may be used to trigger release of reagents from the microcapsules, or from internal compartments therein. In some cases, a microcapsule is degradable. Generally, the trigger may cause disruption or degradation of the shell or membrane enveloping the microcapsule, disruption or degradation of the interior of a microcapsule, and/or disruption or degradation of any chemical bonds that immobilize a reagent to the microcapsule. Exemplary triggers include but are not limited to: chemical triggers, bulk changes, biological triggers, light triggers, thermal triggers, magnetic triggers, and any combination thereof. See, e.g., Esser-Kahn et al., (2011) *Macromolecules* 44: 5539-5553; Wang et al., (2009) *ChemPhysChem* 10:2405-2409;

A. Chemical Stimuli and Bulk Changes

Numerous chemical triggers may be used to trigger the disruption or degradation of the microcapsules. Examples of these chemical changes may include, but are not limited to pH-mediated changes to the shell wall, disintegration of the shell wall via chemical cleavage of crosslink bonds, triggered depolymerization of the shell wall, and shell wall switching reactions. Bulk changes may also be used to trigger disruption of the microcapsules.

A change in pH of the solution, particularly a decrease in pH, may trigger disruption via a number of different mechanisms. The addition of acid may cause degradation or disassembly of the shell wall through a variety of mechanisms. Addition of protons may disassemble cross-linking of polymers in the shell wall, disrupt ionic or hydrogen bonds in the shell wall, or create nanopores in the shell wall to allow the inner contents to leak through to the exterior. In some examples, the microcapsule comprises acid-degradable chemical cross-linkers such a ketals. A decrease in pH, particular to a pH lower than 5, may induce the ketal to convert to a ketone and two alcohols and facilitate disruption of the microcapsule. In other examples, the microcapsules may comprise one or more polyelectrolytes (e.g., PAA, PAAm, PSS, etc.) that are pH sensitive. A decrease in pH may disrupt the ionic- or hydrogen-bonding interactions of such microcapsules, or create nanopores therein. In some cases, microcapsules comprising polyelectrolytes comprise a charged, gel-based core that expands and contracts upon a change of pH.

Removal of cross-linkers (e.g., disulfide bonds) within the microcapsules can also be accomplished through a number of mechanisms. In some examples, various chemicals can be added to a solution of microcapsules that induce either oxidation, reduction or other chemical changes to polymer components of the shell wall. In some cases, a reducing agent, such as beta-mercaptoethanol, dithiotheritol (DTT), or 2-tris(2-carboxyethyl)phosphine (TCEP), is added such that disulfide bonds in a microcapsule shell wall are disrupted. In addition, enzymes may be added to cleave peptide bonds within the microcapsules, thereby resulting in cleavage of shell wall cross linkers.

Depolymerization can also be used to disrupt the microcapsules. A chemical trigger may be added to facilitate the removal of a protecting head group. For example, the trigger may cause removal of a head group of a carbonate ester or carbamate within a polymer, which in turn causes depolymerization and release of reagents from the inside of the capsule.

Shell wall switching reactions may be due to any structural change to the porosity of the shell wall. The porosity of a shell wall may be modified, for example, by the addition of azo dyes or viologen derivatives. Addition of energy (e.g., electricity, light) may also be used to stimulate a change in porosity.

In yet another example, a chemical trigger may comprise an osmotic trigger, whereby a change in ion or solute concentration of microcapsule solution induces swelling of the capsule. Swelling may cause a buildup of internal pressure such that the capsule ruptures to release its contents.

It is also known in the art that bulk or physical changes to the microcapsule through various stimuli also offer many advantages in designing capsules to release reagents. Bulk or physical changes occur on a macroscopic scale, in which capsule rupture is the result of mechano-physical forces induced by a stimulus. These processes may include, but are not limited to pressure induced rupture, shell wall melting, or changes in the porosity of the shell wall.

B. Biological Stimuli

Biological stimuli may also be used to trigger disruption or degradation of microcapsules. Generally, biological triggers resemble chemical triggers, but many examples use biomolecules, or molecules commonly found in living systems such as enzymes, peptides, saccharides, fatty acids, nucleic acids and the like. For example, microcapsules may comprise polymers with peptide cross-links that are sensitive to cleavage by specific proteases. More specifically, one example may comprise a microcapsule comprising GFLGK peptide cross links. Upon addition of a biological trigger such as the protease Cathepsin B, the peptide cross links of the shell well are cleaved and the contents of the capsule are released. In other cases, the proteases may be heat-activated. In another example, microcapsules comprise a shell wall comprising cellulose. Addition of the hydrolytic enzyme

chitosan serves as biologic trigger for cleavage of cellulosic bonds, depolymerization of the shell wall, and release of its inner contents.

C. Thermal Stimuli

The microcapsules may also be induced to release their contents upon the application of a thermal stimulus. A change in temperature can cause a variety changes to the microcapsule. A change in heat may cause melting of a microcapsule such that the shell wall disintegrates. In other cases, the heat may increase the internal pressure of the inner components of the capsule such that the capsule ruptures or explodes. In still other cases, the heat may transform the capsule into a shrunken dehydrated state. The heat may also act upon heat-sensitive polymers within the shell of a microcapsule to cause disruption of the microcapsule.

In one example, a microcapsule comprises a thermo-sensitive hydrogel shell encapsulating one or more emulsified reagent particles. Upon the application of heat, such as above 35 C, the hydrogel material of the outer shell wall shrinks. The sudden shrinkage of the shell ruptures the capsule and allows the reagents of the inside of the capsule to squirt out in the sample preparation solution in the microwell.

In some cases, the shell wall may comprise a diblock polymer, or a mixture of two polymers, with different heat sensitivities. One polymer may be particularly likely to shrink after the application of heat, while the other is more heat-stable. When heat is applied to such shell wall, the heat-sensitive polymer may shrink, while the other remains intact, causing a pore to form. In still other cases, a shell wall may comprise magnetic nanoparticles. Exposure to a magnetic field may cause the generation of heat, leading to rupture of the microcapsule.

D. Magnetic Stimuli

Inclusion of magnetic nanoparticles to the shell wall of microcapsules may allow triggered rupture of the capsules as well as guide the particles in an array. A device of this disclosure may comprise magnetic particles for either purpose. In one example, incorporation of Fe_3O_4 nanoparticles into polyelectrolyte containing capsules triggers rupture in the presence of an oscillating magnetic field stimulus.

E. Electrical and Light Stimuli

A microcapsule may also be disrupted or degraded as the result of electrical stimulation. Similar to magnetic particles described in the previous section, electrically sensitive particles can allow for both triggered rupture of the capsules as well as other functions such as alignment in an electric field, electrical conductivity or redox reactions. In one example, microcapsules containing electrically sensitive material are aligned in an electric field such that release of inner reagents can be controlled. In other examples, electrical fields may induce redox reactions within the shell wall itself that may increase porosity.

A light stimulus may also be used to disrupt the microcapsules. Numerous light triggers are possible and may include systems that use various molecules such as nanoparticles and chromophores capable of absorbing photons of specific ranges of wavelengths. For example, metal oxide coatings can be used as capsule triggers. UV irradiation of polyelectrolyte capsules coated with SiO_2/TiO_2 may result in disintegration of the capsule wall. In yet another example, photo switchable materials such as azobenzene groups may be incorporated in the shell wall. Upon the application of UV or visible light, chemicals such as these undergo a reversible cis-to-trans isomerization upon absorption of photons. In this aspect, incorporation of photo switches result in a shell wall that may disintegrate or become more porous upon the application of a light trigger.

F. Application of Stimuli

A device of this disclosure may be used in combination with any apparatus or device that provides such trigger or stimulus. For example, if the stimulus is thermal, a device may be used in combination with a heated or thermally controlled plate, which allows heating of the microwells and may induce the rupture of capsules. Any of a number of heat transfers may be used for thermal stimuli, including but not limited to applying heat by radiative heat transfer, convective heat transfer, or conductive heat transfer. In other cases, if the stimulus is a biological enzyme, the enzyme may be injected into a device such that it is deposited into each microwell. In another aspect, if the stimulus is a magnetic or electric field, a device may be used in combination with a magnetic or electric plate.

A chemical stimulus may be added to a partition and may exert its function at various times after contacting a chemical stimulus with a microcapsule. The speed at which a chemical stimulus exerts its effect may vary depending on, for example, the amount/concentration of a chemical stimulus contacted with a microcapsule and/or the particular chemical stimulus used. For example, a droplet may comprise a degradable gel bead (e.g., a gel bead comprising chemical cross-linkers, such as, for example, disulfide bonds). Upon droplet formation, a chemical stimulus (e.g., a reducing agent) may be included in the droplet with the gel bead. The chemical stimulus may degrade the gel bead immediately on contact with the gel bead, soon after (e.g., about 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 min) contact with the gel bead, or at a later time. In some cases, degradation of the gel bead may occur before, during, or after a further processing step, such as, for example, a thermal cycling step as described herein.

V. Sample Preparation, Reaction and Recovery

After application of the stimulus, rupturing of capsules and release of the reagents, the sample preparation reaction may proceed in a device. Reactions within a device may be incubated for various periods of times depending on the reagents used in the sample reactions. A device may also be used in combination with other devices that aid in the sample preparation reaction. For example, if PCR amplification is desired, a device may be used in combination with a PCR thermocycler. In some cases, a thermocycler may comprise a plurality of wells. In cases where partitions are droplets, the droplets may be entered into the wells of the thermocycler. In some cases, each well may comprise multiple droplets, such that when thermal cycling is initiated, multiple droplets are thermal cycled in each well. In another example, if the reaction requires agitation, a device may be used in combination with a shaking apparatus.

Following the completion of the sample preparation reaction, the analytes and products of the sample reactions may be recovered. In some cases, a device may utilize a method comprising the application of liquid or gas to flush out the contents of the individual microwells. In one example, the liquid comprises an immiscible carrier fluid that preferentially wets the microwell array material. It may also be immiscible with water so as to flush the reaction products out of the well. In another example, the liquid may be an aqueous fluid that can be used to flush out the samples out of the wells. After flushing of the contents of the microwells, the contents of the microwells are pooled for a variety of downstream analyses and applications.

VI. Applications

FIG. **4**A provides a general flow of many of the methods of the present disclosure; and FIG. **4**B provides a generally annotated version of **4**A. One or more microcapsule(s) that contain reagents **410** may be pre-loaded into microwells, followed by addition of an analyte, which, in this particular Figure, is a nucleic acid analyte **420**. The microwells may then be sealed **430** by any method, such as by application of a sealing fluid. The inlet and outlet ports may also be sealed, for example to prevent evaporation. Following these steps, a stimulus (e.g., heat, chemical, biological, etc.) may be applied to the microwells in order to disrupt the microcapsules **460** and trigger release of the reagents **450** to the interior of the microwell. Subsequently, an incubation step **440** may occur in order to enable the reagents perform a particular function such as lysis of cells, digestion of protein, fragmentation of high molecular weight nucleic acids, or ligation of oligonucleotide bar codes. Following the incubation step (which is optional), the contents of the microwells may be recovered either singly or in bulk.

A. Analytes

A device of this disclosure may have a wide variety of uses in the manipulation, preparation, identification and/or quantification of analytes. In some cases, the analyte is a cell or population of cells. The population of cells may be homogeneous (e.g., from a cell line, of the same cell type, from the same type of tissue, from the same organ, etc.) or heterogenous (mixture of different types of cells). The cells may be primary cells, cell lines, recombinant cells, primary cells, encapsulated cells, free cells, etc.

The analytes may also be molecules, including but not limited to: polypeptides, proteins, antibodies, enzymes, nucleic acids, saccharides, small molecules, drugs, and the like. Examples of nucleic acids include but are not limited to: DNA, RNA, dNTPs, ddNTPs, amplicons, synthetic nucleotides, synthetic polynucleotides, polynucleotides, oligonucleotides, peptide nucleic acids, cDNA, dsDNA, ssDNA, plasmid DNA, cosmid DNA, high Molecular Weight (MW) DNA, chromosomal DNA, genomic DNA, viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), mRNA, rRNA, tRNA, nRNA, siRNA, snRNA, snoRNA, scaRNA, microRNA, dsRNA, ribozyme, riboswitch and viral RNA (e.g., retroviral RNA).

In some cases, the analytes are pre-mixed with one or more additional materials, such as one or more reagents (e.g., ligase, protease, polymerase) prior to being loaded into the device. In some cases, the analytes are pre-mixed with microcapsules comprising one or more reagents prior to being loaded onto the device.

The samples may be derived from a variety of sources including human, mammal, non-human mammal, ape, monkey, chimpanzee, plant, reptilian, amphibian, avian, fungal, viral or bacterial sources. Samples such as cells, nucleic acids and proteins may also be obtained from a variety of clinical sources such as biopsies, aspirates, blood draws, urine samples, formalin fixed embedded tissues and the like.

A device of this disclosure may also enable the analytes to be tagged or tracked in order to permit subsequent identification of an origin of the analytes. This feature is in contrast with other methods that use pooled or multiplex reactions and that only provide measurements or analyses as an average of multiple samples. Here, the physical partitioning and assignment of a unique identifier to individual analytes allows acquisition of data from individual samples and is not limited to averages of samples.

In some examples, nucleic acids or other molecules derived from a single cell may share a common tag or identifier and therefore may be later identified as being derived from that cell. Similarly, all of the fragments from a single strand of nucleic acid may be tagged with the same identifier or tag, thereby permitting subsequent identification of fragments with similar phasing or linkage on the same strand. In other cases, gene expression products (e.g., mRNA, protein) from an individual cell may be tagged in order to quantify expression. In still other cases, the device can be used as a PCR amplification control. In such cases, multiple amplification products from a PCR reaction can be tagged with the same tag or identifier. If the products are later sequenced and demonstrate sequence differences, differences among products with the same identifier can then be attributed to PCR error.

The analytes may be loaded onto the device before, after, or during loading of the microcapsules and/or free reagents. In some cases, the analytes are encapsulated into microcapsules before loading into the microcapsule array. For example, nucleic acid analytes may be encapsulated into a microcapsule, which is then loaded onto the device and later triggered to release the analytes into an appropriate microwell.

Any analytes, such as DNA or cells, may be loaded in solution or as analytes encapsulated in a capsule. In some cases, homogeneous or heterogeneous populations of molecules (e.g., nucleic acids, proteins, etc.) are encapsulated into microcapsules and loaded onto the device. In some cases, homogeneous or heterogeneous populations of cells are encapsulated into microcapsules and loaded onto the device. The microcapsules may comprise a random or specified number of cells and/or molecules. For example, the microcapsules may comprise no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500, 1000, 5000, or 10000 cells and/or molecules per microcapsule. In other examples, the microcapsules comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500, 1000, 5000, or 10000 cells and/or molecules per microcapsule. Fluidic techniques and any other techniques may be used to encapsulate the cells and/or molecules into the microcapsules.

Generally, the methods and compositions provided herein are useful for preparation of an analyte prior to a downstream application such as a sequencing reaction. Often, a sequencing method is classic Sanger sequencing. Sequencing methods may include, but are not limited to: high-throughput sequencing, pyrosequencing, sequencing-by-synthesis, single-molecule sequencing, nanopore sequencing, sequencing-by-ligation, sequencing-by-hybridization, RNA-Seq (Illumina), Digital Gene Expression (Helicos), Next generation sequencing, Single Molecule Sequencing by Synthesis (SMSS)(Helicos), massively-parallel sequencing, Clonal Single Molecule Array (Solexa), shotgun sequencing, Maxim-Gilbert sequencing, primer walking, and any other sequencing methods known in the art.

There are numerous examples of applications that may be conducted instead of, or in conjunction with, a sequencing reaction, including but not limited to: biochemical analyses, proteomics, immunoassays, profiling/fingerprinting of specific cell types, pharmaceutical screening, bait-capture experiments, protein-protein interaction screens and the like.

B. Assignment of Unique Identifiers to Analytes

The devices disclosed herein may be used in applications that involve the assignment of unique identifiers, or molecular bar codes, to analytes. Often, the unique identifier is a bar-code oligonucleotide that is used to tag the analytes; but, in some cases, different unique identifiers are used. For

example, in some cases, the unique identifier is an antibody, in which case the attachment may comprise a binding reaction between the antibody and the analyte (e.g., antibody and cell, antibody and protein, antibody and nucleic acid). In other cases, the unique identifier is a dye, in which case the attachment may comprise intercalation of the dye into the analyte molecule (such as intercalation into DNA or RNA) or binding to a probe labeled with the dye. In still other cases, the unique identifier may be a nucleic acid probe, in which case the attachment to the analyte may comprise a hybridization reaction between the nucleic acid and the analyte. In some cases, the reaction may comprise a chemical linkage between the identifier and the analyte. In other cases, the reaction may comprise addition of a metal isotope, either directly to the analyte or by a probe labeled with the isotope.

Often, the method comprises attaching oligonucleotide bar codes to nucleic acid analytes through an enzymatic reaction such as a ligation reaction. For example, the ligase enzyme may covalently attach a DNA bar code to fragmented DNA (e.g., high molecular-weight DNA). Following the attachment of the bar-codes, the molecules may be subjected to a sequencing reaction.

However, other reactions may be used as well. For example, oligonucleotide primers containing bar code sequences may be used in amplification reactions (e.g., PCR, qPCR, reverse-transcriptase PCR, digital PCR, etc.) of the DNA template analytes, thereby producing tagged analytes. After assignment of bar codes to individual analytes, the contents of individual microwells may be recovered via the outlet port in the device for further analyses.

The unique identifiers (e.g., oligonucleotide bar-codes, antibodies, probes, etc.) may be introduced to the device randomly or nonrandomly. In some cases, they are introduced at an expected ratio of unique identifiers to microwells. For example, the unique identifiers may be loaded so that more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 unique identifiers are loaded per microwell. In some cases, the unique identifiers may be loaded so that less than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 unique identifiers are loaded per microwell. In some cases, the average number of unique identifiers loaded per microwell is less than, or greater than, about 0.0001, 0.001, 0.01, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 unique identifiers per microwell.

The unique identifiers also may be loaded so that a set of one or more identical identifiers are introduced to a particular well. Such sets may also be loaded so that each microwell contains a different set of identifiers. For example, a population of microcapsules may be prepared such that a first microcapsule in the population comprises multiple copies of identical unique identifiers (e.g., nucleic acid bar codes, etc.) and a second microcapsule in the population comprises multiple copies of a unique identifier that differs from within the first microcapsule. In some cases, the population of microcapsules may comprise multiple microcapsules (e.g., greater than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules), each containing multiple copies of a unique identifier that differs from that contained in the other microcapsules. In some cases, the population may comprise greater than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules with identical sets of unique identifiers. In some cases, the population may comprise greater than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules, wherein the microcapsules each comprise a different combination of unique identifiers. For example, in some cases the different combinations overlap, such that a first microcapsule may comprise, e.g., unique identifiers A, B, and C, while a second microcapsule may comprise unique identifiers A, B, and D. In another example, the different combinations do not overlap, such that a first microcapsule may comprise, e.g., unique identifiers A, B, and C, while a second microcapsule may comprise unique identifiers D, E, and F.

The unique identifiers may be loaded into the device at an expected or predicted ratio of unique identifiers per analyte (e.g., strand of nucleic acid, fragment of nucleic acid, protein, cell, etc.) In some cases, the unique identifiers are loaded in the microwells so that more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 unique identifiers are loaded per individual analyte in the microwell. In some cases, the unique identifiers are loaded in the microwells so that less than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 unique identifiers are loaded per individual analyte in the microwell. In some cases, the average number of unique identifiers loaded per analyte is less than, or greater than, about 0.0001, 0.001, 0.01, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 unique identifiers per analyte. When more than one identifier is present per analyte, such identifiers may be copies of the same identifier, or multiple different identifiers. For example, the attachment process may be designed to attach multiple identical identifiers to a single analyte, or multiple different identifiers to the analyte.

The unique identifiers may be used to tag a wide range of analytes, including cells or molecules. For example, unique identifiers (e.g., bar code oligonucleotides) may be attached to whole strands of nucleic acids or to fragments of nucleic acids (e.g., fragmented genomic DNA, fragmented RNA). The unique identifiers (e.g., antibodies, oligonucleotides) may also bind to cells, include the external surface of a cell, a marker expressed on the cell or components within the cell such as organelles, gene expression products, genomic DNA, mitochondrial DNA, RNA, mRNA, or proteins. The unique identifiers also may be designed to bind or hybridize nucleic acids (e.g., DNA, RNA) present in permeabilized cells, which may or may not be otherwise intact.

The unique identifiers may be loaded onto the device either singly or in combination with other elements (e.g., reagents, analytes). In some cases, free unique identifiers are pooled with the analytes and the mixture is loaded into the device. In some cases, unique identifiers encapsulated in microcapsules are pooled with the analytes, prior to loading of the mixture onto the device. In still other cases, free unique identifiers are loaded into the microwells prior to, during (e.g., by separate inlet port), or following the loading of the analytes. In still other cases, unique identifiers encapsulated in microcapsules are loaded into the microwells prior to, concurrently with (e.g., by separate inlet port), or after loading of the analytes.

In many applications, it may be important to determine whether individual analytes each receive a different unique identifier (e.g., oligonucleotide bar code). If the population of unique identifiers introduced into the device is not significantly diverse, different analytes may possibly be tagged with identical identifiers. The devices disclosed herein may enable detection of analytes tagged with the same identifier. In some cases, a reference analyte may be included with the

population of analytes introduced into the device. The reference analyte may be, for example, a nucleic acid with a known sequence and a known quantity. After the population of analytes is loaded and partitioned in the device, unique identifiers may be attached to the analytes, as described herein. If the unique identifiers are oligonucleotide bar codes and the analytes are nucleic acids, the tagged analytes may subsequently be sequenced and quantified. These methods may indicate if one or more fragments and/or analytes may have been assigned an identical bar code.

A method disclosed herein may comprise loading the device with the reagents necessary for the assignment of bar codes to the analytes. In the case of ligation reactions, reagents including, but not limited to, ligase enzyme, buffer, adapter oligonucleotides, a plurality of unique identifier DNA bar codes and the like may be loaded into the device. In the case of enrichment, reagents including but not limited to a plurality of PCR primers, oligonucleotides containing unique identifying sequence, or bar code sequence, DNA polymerase, DNTPs, and buffer and the like may be loaded into the device. The reagents may be loaded as free reagents or as reagents encapsulated in microcapsules.

C. Nucleic Acid Sequencing

Nucleic acid sequencing may begin with the physical partitioning of sample analytes into microwells at a particular density (e.g., about 1 analyte per microwell or other density described herein). When nucleic acid bar codes are assigned to individual analytes, it may then be possible to track individual molecules during subsequent steps such as subsequent amplification and/or sequencing steps, even if the analytes are later pooled together and treated en masse.

a. Nucleic Acid Phasing

The devices provided herein may be used to prepare analytes (e.g., nucleic acid analytes) in such a manner that enables phasing or linkage information to be subsequently obtained. Such information may allow for the detection of linked genetic variations in sequences, including genetic variations (e.g., SNPs, mutations, indels, copy number variations, transversions, translocations, inversions, etc.) that are separated by long stretches of nucleic acids. These variations may exist in either a cis or trans relationship. In cis relationships, two or more genetic variations may exist in the same polynucleic acid molecule or strand. In trans relationships, two or more genetic variations may exist on multiple nucleic acid molecules or strands.

A method of determining nucleic acid phasing may comprise loading a nucleic acid sample (e.g., a nucleic acid sample that spans a given locus or loci) into a device disclosed herein, distributing the sample such that at most one molecule of nucleic acid is present per microwell, and fragmenting the sample within the microwells. The method may further comprise attaching unique identifiers (e.g., bar codes) to the fragmented nucleic acids as described herein, recovering the nucleic acids in bulk, and performing a subsequent sequencing reaction on the samples in order to detect genetic variations, such as two different genetic variations. The detection of genetic variations tagged with two different bar codes may indicate that the two genetic variations are derived from two separate strands of DNA, reflecting a trans relationship. Conversely, the detection of two different genetic variations tagged with the same bar codes may indicate that the two genetic variations are from the same strand of DNA, reflecting a cis relationship.

Phase information may be important for the characterization of the analyte, particularly if the analyte derives from a subject at risk of, having, or suspected of a having a particular disease or disorder (e.g., hereditary recessive disease such as Cystic Fibrosis, cancer, etc.). The information may be able to distinguish between the following possibilities: (1) two genetic variations within the same gene on the same strand of DNA and (2) two genetic variations within the same gene but located on separate strands of DNA. Possibility (1) may indicate that one copy of the gene is normal and the individual is free of the disease, while possibility (2) may indicate that the individual has or will develop the disease, particularly if the two genetic variations are damaging to the function of the gene when present within the same gene copy. Similarly, the phasing information may also be able to distinguish between the following possibilities: (1) two genetic variations, each within a different gene on the same strand of DNA and (2) two genetic variations, each within a different gene but located on separate strands of DNA.

b. Cell-Specific Information

The devices provided herein may be used to prepare cellular analytes in such a manner that enables cell-specific information to be subsequently obtained. Such information may enable detection of genetic variations (e.g., SNPs, mutations, indels, copy number variations, transversions, translocations, inversions, etc.) on a cell-by-cell basis, thereby enabling a determination of whether the genetic variation(s) are present in the same cell or two different cells.

A method of determining nucleic acid cell-specific information may comprise loading a cellular sample (e.g., a cellular sample from a subject) into a device disclosed herein, distributing the sample such that at most one cell is present per microwell, lysing the cells, and then tagging the nucleic acids within the cells with unique identifiers using a method described herein. In some cases, microcapsules comprising unique identifiers are loaded in the microwell array device (either before, during, or after the loading of the cellular analytes) in such a manner that each cell is contacted with a different microcapsule. The resulting tagged nucleic acids can then be pooled, sequenced, and used to trace the origin of the nucleic acids. Nucleic acids with identical unique identifiers may be determined to originate from the same cell, while nucleic acids with different unique identifiers may be determined to originate from different cells.

In a more specific example, the methods herein may be used to detect the distribution of oncogenic mutations across a population of cancer tumor cells. In this example, some of the cells may have a mutation, or amplification, of an oncogene (e.g., HER2, BRAF, EGFR, KRAS) on two strands of DNA (homozygous), while others may be heterozygous for the mutation, while still other cells may be wild-type and comprise no mutations or other variation in the oncogene. The methods described herein may be able to detect these differences, and also may enable quantification of the relative numbers of homozygous, heterozygous, and wild-type cells. Such information may be used to stage a particular cancer or to monitor the progression of the cancer over time.

In some examples, this disclosure provides methods of identifying mutations in two different oncogenes (e.g., KRAS and EGFR). If the same cell comprises genes with both mutations, this may indicate a more aggressive form of cancer. In contrast, if the mutations are located in two different cells, this may indicate that the cancer is more benign, or less advanced.

The following is another specific example of cell-specific sequence determination. In this example, a plurality of cells, such as from a tumor biopsy, is loaded into a device. Single cells from the sample are deposited into individual wells and labeled with a DNA bar code.

Loading of cells into a device may be achieved through non-random loading. Parameters for non-random loading of analytes, such as cells, may be understood using an interference function such that:

$$\text{“fraction multi-occupancy”} = 1 - \left[\left(1 - \frac{1}{N}\right) + \frac{P}{N}\right]^{C}$$

where

P=probability that a particular cell will attempt but not fit in the well (measure of interference)

N=number of wells

L=number of labels=barcodes

C=number of cells

As part of sample preparation reactions, cells may be lysed and many subsequent reactions are possible, including RNA amplification, DNA amplification or antibody screening for different target proteins and genes in individual cells. After the reaction, the contents of the cells may be pooled together and could be further analyzed, such as by DNA sequencing. With each cell assigned a unique barcode, further analyses may be possible including but not limited to quantification of different gene levels or nucleic acid sequencing of individual cells. In this example, it may be determined whether the tumor comprises cells with different genetic backgrounds (e.g., cancer clones and subclones). The relative number of each type of cell may also be calculated.

c. Amplification Control

As disclosed herein, the device can be used for purposes of controlling for amplification errors, such as PCR errors. For example, a nucleic acid sample may be partitioned into the microwells of the device. Following partitioning, the sample may be subjected to a PCR amplification reaction within the microwells. The PCR products within a microwell may be tagged with the same unique identifier, using a method described herein. If the products are later sequenced and demonstrate sequence differences, differences among products with the same identifier can then be attributed to PCR error.

d. Gene-expression Products Analysis

In other applications, a device may be used to detect gene product (e.g., protein, mRNA) expression levels in a sample, often on a cell-by-cell basis. A sample may comprise individual cells, a pool of mRNA extract from cells, or other collection of gene products. In some instances, single cells may be loaded into microwells. In other instances, a pool of mRNA or other gene product may be loaded such that a desired quantity of mRNA molecules is loaded into individual microwells.

The methods provided herein may be particularly useful for RNA analysis. For example, using the methods provided herein, unique identifiers may be assigned to mRNA analytes either directly or to cDNA products of a reverse transcription reaction performed on the mRNA analytes. The reverse transcription reaction may be conducted within the microwells of the device following loading of the analytes. Reagents for the reaction may include but are not limited to reverse transcriptase, DNA polymerase enzyme, buffer, dNTPs, oligonucleotide primers, oligonucleotide primers containing bar code sequences and the like. One or more reagents may be loaded into microcapsules or loaded freely in solution into the device or a combination thereof. Sample preparation may then be conducted, such as by fragmenting the cDNA and attaching unique identifiers to the fragments. After sample preparation and recovery, the nucleic acid products of the reaction may be further analyzed, such as by sequencing.

Additionally, a device may be used to characterize multiple cell markers, similar to a flow cytometer. Any cell marker may be characterized, including cell-surface markers (e.g., extracellular proteins, transmembrane markers) and markers located within the internal portion of a cell (e.g., RNA, mRNA, microRNA, multiple copies of genes, proteins, alternative splicing products, etc.). For example, cells may be partitioned within the device, as described herein, so that at most one cell is present within a microwell. Cell markers such as nucleic acids (e.g., RNA) may be extracted and/or fragmented prior to being labeled with a unique identifier (e.g., molecular bar code). Or, alternatively, the nucleic acids may be labeled with a unique identifier without being extracted and/or fragmented. The nucleic acids may then be subjected to further analysis such as sequencing reactions designed to detect multiple gene expression products. Such analysis may be useful in a number of fields. For example, if the starting cells are immune cells (e.g., T cells, B cells, macrophages, etc.), the analysis may provide information regarding multiple expressed markers and enable immunophenotyping of the cells, for example by identifying different CD markers of the cells (e.g., CD3, CD4, CD8, CD19, CD20, CD 56, etc.). Such markers can provide insights into the function, character, class, or relative maturity of the cell. Such markers can also be used in conjunction with markers that are not necessarily immunophenotyping markers, such as markers of pathogenic infection (e.g., viral or bacterial protein, DNA, or RNA). In some cases, the device may be used to identify at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 500, 700, 1000, 5000, 10000, 50000, or 100000 different gene expression products or other form of cellular markers on a single-cell basis. Often, such methods do not comprise use of dyes or probes (e.g., fluorescent probes or dyes).

Gene expression product analysis may be useful in numerous fields including immunology, cancer biology (e.g., to characterize the existence, type, stage, aggressiveness, or other characteristic of cancerous tissue), stem cell biology (e.g., in order to characterize the differentiation state of a stem cell, potency of a stem cell, cellular type of a stem cell, or other features of a stem cell), microbiology, and others. The gene expression analysis may also be used in drug screening applications, for example to evaluate the effect of a particular drug or agent on the gene expression profile of particular cells.

VII. Terminology

The terminology used therein is for the purpose of describing particular embodiments only and is not intended to be limiting of a device of this disclosure. As used herein, the singular forms “a”, “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms “including”, “includes”, “having”, “has”, “with”, or variants thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner similar to the term “comprising”.

Several aspects of a device of this disclosure are described above with reference to example applications for illustration. It should be understood that numerous specific details, relationships, and methods are set forth to provide a full understanding of a device. One having ordinary skill in the relevant art, however, will readily recognize that a device

can be practiced without one or more of the specific details or with other methods. This disclosure is not limited by the illustrated ordering of acts or events, as some acts may occur in different orders and/or concurrently with other acts or events. Furthermore, not all illustrated acts or events are required to implement a methodology in accordance with this disclosure.

Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. The term "about" as used herein refers to a range that is 15% plus or minus from a stated numerical value within the context of the particular usage. For example, about 10 would include a range from 8.5 to 11.5.

The term microwell array, as used herein, generally refers to a predetermined spatial arrangement of microwells. Microwell array devices that comprise a microcapsule may also be referred to as "microwell capsule arrays." Further, the term "array" may be used herein to refer to multiple arrays arranged on a surface, such as would be the case where a surface has multiple copies of an array. Such surfaces bearing multiple arrays may also be referred to as "multiple arrays" or "repeating arrays."

EXAMPLE 1

Single Cell DNA Sequencing

A microwell capsule array is prepared to perform nucleic acid sequencing on individual human B-cells taken from a blood sample. Approximately 15,000 cells are harvested and used for loading into the device. A device of this disclosure and containing 150,000 microwells is used. Each well is cylindrical in shape having a diameter of 125 um and a height of 125 um, allowing at most 1 capsule to be loaded per well. Microcapsules made through emulsion polymerization with a PNIPAM hydrogel shell wall are created such that the microcapsules have a diameter of 100 um for loading in the device. The microcapsules are created such that the PNIPAM shell contains magnetic iron particles. The outer surface of the shell is then chemically coupled to a antibody specific to a transmembrane B cell receptor on the outside of a B cell.

During the preparation process of capsules, reagents are simultaneously loaded into the capsules. Reagents necessary for cell lysis and labeling individual DNA strands of the cells with DNA barcodes are loaded into capsules. Reagents for cell lysis include a mild non-ionic detergent, buffer and salt. Reagents for the addition of DNA bar codes to genomic DNA included restriction enzymes, ligase, and >10,000,000 unique DNA oligonucleotides are loaded into capsules. Capsules are designed to be sensitive to rupture at greater than 65 C.

Capsules are prepared to be applied to the microcapsule array. The array is placed on a magnetic temperature controlled hot plate. Microcapsules are added to a sample of B cells such that one B cell is able to bind to one capsule. Capsule-cell conjugates are applied in aqueous carrier solution in a quantity in excess to the relative number of wells. Gentle pipetting of capsules-cells into the inlet port followed by application of a vacuum manifold to the outlet port distributes the capsules throughout the device. A magnetic field is applied through the plate. Excess capsule-cell solution is removed via pipetting through the outlet port. Each capsule-cell conjugate is trapped and positioned in individual wells via the magnetic field.

After the cells and capsules are loaded in the device, a carrier oil (or sealing fluid) is applied to the device to remove any excess aqueous solution bridging adjacent microwells. The carrier oil applied to the inlet and excess oil is recovered at the outlet with a vacuum manifold. After the carrier oil is applied, the inlet and outlet ports are sealed with tape.

The device is then heated, via the magnetic temperature controlled hot plate, to a temperature of 70 C for 10 min to allow for capsule rupture and cell lysis. The hot plate is then switched to 37 C, for restriction and ligation, for up to 1 hour.

After the sample preparation reaction is completed, the contents of the wells are recovered. The inlet and outlet ports of the device are unsealed and nitrogen gas is applied to the device to flush out the individual components of the microwells. The sample is collected in bulk via a pipette at the outlet port, while the magnetic field retains ruptured capsule shells in individual microwells.

The sample is then sequenced using a multiplex sequencing strategy known in the art. Bar coding of individual cells allows for sequencing information to be gained for individual cells rather than as an average of multiple cells. Based upon the number of cells sequenced and bar codes assigned, SNP cell-specific information is gained. Moreover, the number of reads for individual bar codes can be counted to provide insight into the distribution of different types of cells with varying genetic backgrounds, within the original population of B cells.

EXAMPLE 2

DNA Single Strand Sequencing

A microwell capsule array is prepared to perform nucleic acid sequencing on individual strands of DNA isolated from a population of human skin cells. Cells are lysed using detergent and heat and approximately 15,000 copies of diploid DNA are precipitated via chloroform/ethanol extraction. A resuspension of DNA is loaded into the device with approximately 10,000 copies of haploid DNA. A device of this disclosure, with 300,000 microwells is used. Each well is cylindrical in shape having a diameter of 125 um and a height of 125 um, allowing at most 1 capsule to be loaded per well. Microcapsules made through emulsion polymerization with a PNIPAM hydrogel shell wall are created to a specification of a sphere with a diameter of 100 um for loading into the device.

During the preparation of the microcapsules, reagents are simultaneously loaded into the capsules. The reagents include reagents necessary for labeling individual DNA strands with DNA barcodes, including restriction enzymes, ligase, and >10,000,000 unique DNA oligonucleotides. Capsules designed to be sensitive to rupture at greater than 65 C are used for the encapsulation.

Capsules are applied aqueous carrier solution in an excess to the relative number of wells. Gentle pipetting of capsules into the inlet followed by application of a vacuum manifold to the outlet distributed the capsules throughout the device.

After excess capsule solution is removed, a suspension of DNA in buffer is applied to the device in a similar fashion as the capsules.

After the DNA strands and capsules are loaded in the device, a carrier oil is applied to the device to remove any excess aqueous solution bridging adjacent microwells. The carrier oil is applied to the inlet port and excess oil is recovered at the outlet port with a vacuum manifold. After the carrier oil is applied, the inlet and outlet ports are sealed with tape.

The device is then placed on a temperature controlled hot plate and heated to temperature of 70 C for 10 min to allow for capsule rupture. Reagents are released into the sample preparation reaction. The hot plate is then switched to 37 C, for restriction and ligation, for up to 1 hour.

After the sample preparation reaction is completed, the inlet and outlet ports of the device are unsealed and nitrogen gas is applied to the device to flush out the individual components of the microwells. The sample products, en bulk, are collected via pipette at the outlet port.

The sample is then sequenced to sufficient coverage (e.g., 500) using a multiplex sequencing strategy known in the art. Bar coding of individual DNA strands allows for sequencing information to be gained from individual strands rather than as an average of entire sample of DNA. Based upon the number of DNA strands sequenced and bar codes assigned, SNP phasing/haplotyping information is gained and many repetitive regions of DNA can be resolved. In addition, a substantial boost in accuracy can be gained by discarding mutations that appear randomly with respect to haplotypes, as those are likely to be sequencing errors.

It should be understood from the foregoing that, while particular implementations have been illustrated and described, various modifications may be made thereto and are contemplated herein. It is also not intended that the invention be limited by the specific examples provided within the specification. While the invention has been described with reference to the aforementioned specification, the descriptions and illustrations of the preferable embodiments herein are not meant to be construed in a limiting sense. Furthermore, it shall be understood that all aspects of the invention are not limited to the specific depictions, configurations or relative proportions set forth herein which depend upon a variety of conditions and variables. Various modifications in form and detail of the embodiments of the invention will be apparent to a person skilled in the art. It is therefore contemplated that the invention shall also cover any such modifications, variations and equivalents. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

What is claimed is:

1. A method for droplet generation, comprising:

(a) providing at least 1,000,000 oligonucleotide molecules comprising barcode sequences, wherein said barcode sequences are the same sequence for said at least 1,000,000 oligonucleotide molecules, wherein said at least 1,000,000 oligonucleotide molecules are releasably attached to a bead, wherein said bead is porous;

(b) combining said at least 1,000,000 oligonucleotide molecules and a sample comprising a nucleic acid analyte each in an aqueous phase at a first junction of two or more channels of a microfluidic device to form an aqueous mixture comprising said at least 1,000,000 oligonucleotide molecules attached to said bead and said sample; and

(c) generating a droplet comprising said at least 1,000,000oligonucleotide molecules attached to said bead and said sample comprising said nucleic acid analyte by contacting said aqueous mixture with an immiscible continuous phase at a second junction of two or more channels of said microfluidic device.

2. The method of claim **1**, further comprising, in (b), combining said at least 1,000,000 oligonucleotide molecules attached to said bead, said nucleic acid analyte and one or more reagents necessary for amplification of said nucleic acid analyte at said first junction to form said aqueous mixture comprising said at least 1,000,000 oligonucleotide molecules attached to said bead, said nucleic acid analyte and said one or more reagents.

3. The method of claim **2**, wherein in (c), said droplet further comprises said one or more reagents.

4. The method of claim **3**, wherein said one or more reagents comprises a polymerase.

5. The method of claim **4**, wherein said polymerase is unable to recognize uracil.

6. The method of claim **1**, wherein said bead comprises a polyacrylamide.

7. The method of claim **1**, wherein said bead is a gel bead.

8. The method of claim **1**, wherein said at least 1,000,000 oligonucleotide molecules comprise uracil.

9. The method of claim **1**, wherein a given oligonucleotide molecule of said at least 1,000,000 oligonucleotide molecules comprises a region which functions as a primer.

10. The method of claim **9**, wherein said region which functions as said primer has a sequence for random priming.

11. The method of claim **9**, further comprising, after (c), amplifying said nucleic acid analyte with said primer.

12. The method of claim **1**, wherein said nucleic acid analyte is selected from the group consisting of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), amplicons, synthetic polynucleotides, polynucleotides, oligonucleotides, cDNA, dsDNA, ssDNA, plasmid DNA, cosmid DNA, High Molecular Weight (MW) DNA, chromosomal DNA, genomic DNA, viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), mRNA, rRNA, tRNA, nRNA, siRNA, snRNA, snoRNA, scaRNA, microRNA, dsRNA, ribozyme, riboswitch and viral RNA.

13. The method of claim **1**, wherein said at least 1,000,000 oligonucleotide molecules are attached to said bead via a chemical cross-linker.

14. The method of claim **1**, wherein said at least 1,000,000 oligonucleotide molecules are attached to said bead via a disulfide bond.

15. The method of claim **1**, wherein said at least 1,000,000 oligonucleotide molecules are attached to said bead via a covalent bond.

16. The method of claim **1**, wherein said at least 1,000,000 oligonucleotide molecules are attached to said bead via a labile moiety.

17. The method of claim **1**, wherein said bead is degradable upon application of a stimulus.

18. The method of claim **17**, further comprising applying said stimulus to the droplet to release said at least 1,000,000 oligonucleotide molecules from said bead into said droplet.

19. The method of claim **18**, wherein said stimulus is selected from the group consisting of a biological stimulus, a chemical stimulus, a thermal stimulus, an electrical stimulus, a magnetic stimulus, and a photo stimulus.

20. The method of claim **19**, wherein said stimulus is a chemical stimulus that is a reducing agent.

21. The method of claim **1**, wherein subsequent to generating said droplet in (c), a given oligonucleotide molecule of said at least 1,000,000 oligonucleotide molecules attaches to said nucleic acid analyte, and wherein said given oligonucleotide molecule attached to said given nucleic acid analyte is subjected to nucleic acid amplification to yield a barcoded nucleic acid analyte.

22. The method of claim **1**, wherein said bead comprises a chemical cross-linker.

23. The method of claim **22**, wherein said chemical cross-linker is a disulfide bond.

* * * * *

JX-0007


US009856530B2

(12) **United States Patent**
Hindson et al.

(10) **Patent No.: US 9,856,530 B2**
(45) **Date of Patent: *Jan. 2, 2018**

(54) **METHODS AND SYSTEMS FOR PROCESSING POLYNUCLEOTIDES**

(71) Applicant: **10X Genomics, Inc.**, Pleasanton, CA (US)

(72) Inventors: **Benjamin Hindson**, Pleasanton, CA (US); **Serge Saxonov**, Oakland, CA (US); **Kevin Ness**, Boulder, CO (US); **Paul Hardenbol**, San Francisco, CA (US); **Michael Schnall-Levin**, San Francisco, CA (US); **Mirna Jarosz**, Palo Alto, CA (US)

(73) Assignee: **10X GENOMICS, INC.**, Pleasanton, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **15/588,519**

(22) Filed: **May 5, 2017**

(65) **Prior Publication Data**

US 2017/0247757 A1 Aug. 31, 2017

Related U.S. Application Data

(63) Continuation of application No. 15/376,582, filed on Dec. 12, 2016, now Pat. No. 9,701,998, which is a continuation-in-part of application No. 14/104,650, filed on Dec. 12, 2013, now Pat. No. 9,567,631, said application No. 15/376,582 is a continuation-in-part of application No. 14/250,701, filed on Apr. 11, 2014, now abandoned, which is a continuation of application No. 14/175,973, filed on Feb. 7, 2014, now Pat. No. 9,388,465.

(60) Provisional application No. 61/737,374, filed on Dec. 14, 2012, provisional application No. 61/844,804, filed on Jul. 10, 2013, provisional application No. 61/840,403, filed on Jun. 27, 2013, provisional application No. 61/800,223, filed on Mar. 15, 2013, provisional application No. 61/762,435, filed on Feb. 8, 2013.

(51) **Int. Cl.**
C12Q 1/68 (2006.01)

(52) **U.S. Cl.**
CPC ***C12Q 1/6874*** (2013.01); ***C12Q 1/6806*** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

2,797,149 A 6/1957 Skeggs
3,047,367 A 7/1962 Kessler
3,479,141 A 11/1969 William et al.
4,253,846 A 3/1981 Smythe et al.
4,582,802 A 4/1986 Zimmerman et al.
5,149,625 A 9/1992 Church et al.
5,185,099 A 2/1993 Delpuech et al.
5,202,231 A 4/1993 Drmanac et al.
5,270,183 A 12/1993 Corbett et al.
5,413,924 A 5/1995 Kosak et al.
5,436,130 A 7/1995 Mathies et al.
5,489,523 A 2/1996 Mathur
5,512,131 A 4/1996 Kumar et al.
5,585,069 A 12/1996 Zanzucchi et al.
5,587,128 A 12/1996 Wilding et al.
5,605,793 A 2/1997 Stemmer
5,618,711 A 4/1997 Gelfand et al.
5,695,940 A 12/1997 Drmanac et al.
5,708,153 A 1/1998 Dower et al.
5,736,330 A 4/1998 Fulton
5,739,036 A 4/1998 Parris
5,744,311 A 4/1998 Fraiser et al.
5,756,334 A 5/1998 Perler et al.
5,834,197 A 11/1998 Parton
5,842,787 A 12/1998 Kopf-Sill et al.
5,846,719 A 12/1998 Brenner et al.
5,846,727 A 12/1998 Soper et al.
5,851,769 A 12/1998 Gray et al.
5,856,174 A 1/1999 Lipshutz et al.
5,872,010 A 2/1999 Karger et al.
5,958,703 A 9/1999 Dower et al.
5,994,056 A 11/1999 Higuchi
5,997,636 A 12/1999 Gamarnik et al.
6,033,880 A 3/2000 Haff et al.
6,046,003 A 4/2000 Mandecki
6,051,377 A 4/2000 Mandecki
(Continued)

FOREIGN PATENT DOCUMENTS

EP 0249007 A2 12/1987
EP 0271281 A2 6/1988
EP 0637996 B1 7/1997
EP 1019496 B1 9/2004
EP 1672064 A1 6/2006
EP 1482036 B1 10/2007
EP 1841879 A2 10/2007
EP 1594980 B1 11/2009
EP 1967592 B1 4/2010
EP 2258846 A2 12/2010
(Continued)

OTHER PUBLICATIONS

Abate, et al. Beating Poisson encapsulation statistics using close-packed ordering. Lab Chip. Sep. 21, 2009;9(18):2628-31. doi: 10.1039/b909386a. Epub Jul. 28, 2009.
(Continued)

Primary Examiner — David C Thomas
(74) *Attorney, Agent, or Firm* — Wilson Sonsini Goodrich & Rosati

(57) **ABSTRACT**

The present disclosure provides compositions, methods, systems, and devices for polynucleotide processing. Such polynucleotide processing may be useful for a variety of applications, including polynucleotide sequencing.

30 Claims, 8 Drawing Sheets

10XG-0000000114

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,057,107 A 5/2000 Fulton
6,057,149 A 5/2000 Burns et al.
6,103,537 A 8/2000 Ullman et al.
6,143,496 A 11/2000 Brown et al.
6,171,850 B1 1/2001 Nagle et al.
6,172,218 B1 1/2001 Brenner
6,265,552 B1 7/2001 Schatz
6,296,020 B1 10/2001 McNeely et al.
6,297,006 B1 10/2001 Drmanac et al.
6,297,017 B1 10/2001 Schmidt et al.
6,303,343 B1 10/2001 Kopf-Sill
6,306,590 B1 10/2001 Mehta et al.
6,327,410 B1 12/2001 Walt et al.
6,355,198 B1 3/2002 Kim et al.
6,361,950 B1 3/2002 Mandecki
6,372,813 B1 4/2002 Johnson et al.
6,379,929 B1 4/2002 Burns et al.
6,406,848 B1 6/2002 Bridgham et al.
6,409,832 B2 6/2002 Weigl et al.
6,432,290 B1 8/2002 Harrison et al.
6,432,360 B1 8/2002 Church
6,485,944 B1 11/2002 Church et al.
6,492,118 B1 12/2002 Abrams et al.
6,511,803 B1 1/2003 Church et al.
6,524,456 B1 2/2003 Ramsey et al.
6,569,631 B1 5/2003 Pantoliano et al.
6,586,176 B1 7/2003 Trnovsky et al.
6,632,606 B1 10/2003 Ullman et al.
6,632,655 B1 10/2003 Mehta et al.
6,670,133 B2 12/2003 Knapp et al.
6,723,513 B2 4/2004 Lexow
6,767,731 B2 7/2004 Hannah
6,800,298 B1 10/2004 Burdick et al.
6,806,052 B2 10/2004 Bridgham et al.
6,806,058 B2 10/2004 Jesperson et al.
6,859,570 B2 2/2005 Walt et al.
6,880,576 B2 4/2005 Karp et al.
6,884,788 B2 4/2005 Bulpitt et al.
6,913,935 B1 7/2005 Thomas
6,929,859 B2 8/2005 Chandler et al.
6,969,488 B2 11/2005 Bridgham et al.
6,974,669 B2 12/2005 Mirkin et al.
7,041,481 B2 5/2006 Anderson et al.
7,115,400 B1 10/2006 Adessi et al.
7,129,091 B2 10/2006 Ismagilov et al.
7,268,167 B2 9/2007 Higuchi et al.
7,282,370 B2 10/2007 Bridgham et al.
7,294,503 B2 11/2007 Quake et al.
7,297,485 B2 11/2007 Bornarth et al.
7,323,305 B2 1/2008 Leamon et al.
7,329,493 B2 2/2008 Chou et al.
7,425,431 B2 9/2008 Church et al.
7,536,928 B2 5/2009 Kazuno
7,544,473 B2 6/2009 Brenner
7,604,938 B2 10/2009 Takahashi et al.
7,622,280 B2 11/2009 Holliger et al.
7,638,276 B2 12/2009 Griffiths et al.
7,645,596 B2 1/2010 Williams et al.
7,666,664 B2 2/2010 Sarofim et al.
7,700,325 B2 4/2010 Cantor et al.
7,708,949 B2 5/2010 Stone et al.
7,709,197 B2 5/2010 Drmanac
7,745,178 B2 6/2010 Dong
7,776,927 B2 8/2010 Chu et al.
RE41,780 E 9/2010 Anderson et al.
7,799,553 B2 9/2010 Mathies et al.
7,842,457 B2 11/2010 Berka et al.
7,901,891 B2 3/2011 Drmanac
7,910,354 B2 3/2011 Drmanac et al.
7,960,104 B2 6/2011 Drmanac et al.
7,968,287 B2 6/2011 Griffiths et al.
7,972,778 B2 7/2011 Brown et al.
8,003,312 B2 8/2011 Krutzik et al.
8,008,018 B2 8/2011 Quake et al.
8,053,192 B2 11/2011 Bignell et al.
8,067,159 B2 11/2011 Brown et al.
8,101,346 B2 1/2012 Takahama
8,133,719 B2 3/2012 Drmanac et al.
8,168,385 B2 5/2012 Brenner
8,252,539 B2 8/2012 Quake et al.
8,268,564 B2 9/2012 Roth et al.
8,273,573 B2 9/2012 Ismagilov et al.
8,278,071 B2 10/2012 Brown et al.
8,298,767 B2 10/2012 Brenner et al.
8,304,193 B2 11/2012 Ismagilov et al.
8,318,433 B2 11/2012 Brenner
8,318,460 B2 11/2012 Cantor et al.
8,329,407 B2 12/2012 Ismagilov et al.
8,337,778 B2 12/2012 Stone et al.
8,361,299 B2 1/2013 Sabin et al.
8,563,274 B2 10/2013 Brenner et al.
8,592,150 B2 11/2013 Drmanac et al.
8,603,749 B2 12/2013 Gillevet
8,679,756 B1 3/2014 Brenner et al.
8,748,094 B2 6/2014 Weitz et al.
8,748,102 B2 6/2014 Berka et al.
8,765,380 B2 7/2014 Berka et al.
8,822,148 B2 9/2014 Ismagliov et al.
8,835,358 B2 9/2014 Fodor et al.
8,871,444 B2 10/2014 Griffiths et al.
8,889,083 B2 11/2014 Ismagilov et al.
8,986,286 B2 3/2015 Tanghoej et al.
9,012,390 B2 4/2015 Holtze et al.
9,017,948 B2 4/2015 Agresti et al.
9,029,083 B2 5/2015 Griffiths et al.
9,029,085 B2 5/2015 Agresti et al.
9,068,210 B2 6/2015 Agresti et al.
9,102,980 B2 8/2015 Brenner et al.
9,249,460 B2 2/2016 Pushkarev et al.
9,290,808 B2 3/2016 Fodor et al.
9,347,059 B2 5/2016 Saxonov
9,388,465 B2 7/2016 Hindson et al.
9,410,201 B2 * 8/2016 Hindson C12Q 1/6874
9,567,631 B2 2/2017 Hindson et al.
9,644,204 B2 5/2017 Hindson et al.
9,689,024 B2 6/2017 Hindson et al.
9,694,361 B2 7/2017 Bharadwaj et al.
9,695,468 B2 7/2017 Hindson et al.
9,701,998 B2 * 7/2017 Hindson C12Q 1/6806
2001/0020588 A1 9/2001 Adourian et al.
2001/0036669 A1 11/2001 Jedrzejewski et al.
2001/0041357 A1 11/2001 Fouillet et al.
2001/0044109 A1 11/2001 Mandecki
2001/0048900 A1 12/2001 Bardell et al.
2002/0001856 A1 1/2002 Chow et al.
2002/0005354 A1 1/2002 Spence et al.
2002/0034737 A1 3/2002 Drmanac
2002/0043463 A1 4/2002 Shenderov
2002/0051971 A1 5/2002 Stuelpnagel et al.
2002/0051992 A1 5/2002 Bridgham et al.
2002/0058332 A1 5/2002 Quake et al.
2002/0089100 A1 7/2002 Kawasaki
2002/0092767 A1 7/2002 Bjornson et al.
2002/0113009 A1 8/2002 O'Connor et al.
2002/0119536 A1 8/2002 Stern
2002/0131147 A1 9/2002 Paolini et al.
2002/0160518 A1 10/2002 Hayenga et al.
2002/0164820 A1 11/2002 Brown
2002/0166582 A1 11/2002 O'Connor et al.
2002/0175079 A1 11/2002 Christel et al.
2002/0179849 A1 12/2002 Maher et al.
2003/0005967 A1 1/2003 Karp
2003/0007898 A1 1/2003 Bohm et al.
2003/0008285 A1 1/2003 Fischer
2003/0008323 A1 1/2003 Ravkin et al.
2003/0022231 A1 1/2003 Wangh et al.
2003/0027221 A1 2/2003 Scott et al.
2003/0028981 A1 2/2003 Chandler et al.
2003/0036206 A1 2/2003 Chien et al.
2003/0039978 A1 2/2003 Hannah
2003/0044777 A1 3/2003 Beattie
2003/0044836 A1 3/2003 Levine et al.
2003/0075446 A1 4/2003 Culbertson et al.
2003/0082587 A1 5/2003 Seul et al.

(56) **References Cited**

U.S. PATENT DOCUMENTS

2003/0089605 A1 5/2003 Timperman
2003/0104466 A1 6/2003 Knapp et al.
2003/0108897 A1 6/2003 Drmanac
2003/0124509 A1 7/2003 Kenis et al.
2003/0149307 A1 8/2003 Hai et al.
2003/0170698 A1 9/2003 Gascoyne et al.
2003/0182068 A1 9/2003 Battersby et al.
2003/0207260 A1 11/2003 Trnovsky et al.
2003/0215862 A1 11/2003 Parce et al.
2004/0063138 A1 4/2004 McGinnis et al.
2004/0081962 A1 4/2004 Chen et al.
2004/0132122 A1 7/2004 Banerjee et al.
2004/0224331 A1 11/2004 Cantor et al.
2004/0258701 A1 12/2004 Dominowski et al.
2005/0019839 A1 1/2005 Jespersen et al.
2005/0042625 A1 2/2005 Schmidt et al.
2005/0079510 A1 4/2005 Berka et al.
2005/0130188 A1 6/2005 Walt et al.
2005/0172476 A1 8/2005 Stone et al.
2005/0181379 A1 8/2005 Su et al.
2005/0202429 A1 9/2005 Trau et al.
2005/0202489 A1 9/2005 Cho et al.
2005/0221339 A1 10/2005 Griffiths et al.
2005/0244850 A1 11/2005 Huang et al.
2005/0287572 A1 12/2005 Mathies et al.
2006/0002890 A1 1/2006 Hersel et al.
2006/0008799 A1 1/2006 Cai et al.
2006/0020371 A1 1/2006 Ham et al.
2006/0073487 A1 4/2006 Oliver et al.
2006/0078888 A1 4/2006 Griffiths et al.
2006/0153924 A1 7/2006 Griffiths et al.
2006/0163385 A1 7/2006 Link et al.
2006/0177832 A1 8/2006 Brenner
2006/0177833 A1 8/2006 Brenner
2006/0199193 A1 9/2006 Koo et al.
2006/0240506 A1 10/2006 Kushmaro et al.
2006/0257893 A1 11/2006 Takahashi et al.
2006/0263888 A1 11/2006 Fritz et al.
2006/0275782 A1 12/2006 Gunderson et al.
2006/0292583 A1 12/2006 Schneider et al.
2007/0003442 A1 1/2007 Link et al.
2007/0020617 A1 1/2007 Trnovsky et al.
2007/0020640 A1 1/2007 McCloskey et al.
2007/0054119 A1 3/2007 Garstecki et al.
2007/0072208 A1 3/2007 Drmanac
2007/0077572 A1 4/2007 Tawfik et al.
2007/0092914 A1 4/2007 Griffiths et al.
2007/0099208 A1 5/2007 Drmanac et al.
2007/0154903 A1 7/2007 Marla et al.
2007/0160503 A1 7/2007 Sethu et al.
2007/0172873 A1 7/2007 Brenner et al.
2007/0190543 A1 8/2007 Livak
2007/0195127 A1 8/2007 Ahn et al.
2007/0207060 A1 9/2007 Zou et al.
2007/0228588 A1 10/2007 Noritomi et al.
2007/0238113 A1 10/2007 Kanda et al.
2007/0259357 A1 11/2007 Brenner
2007/0264320 A1 11/2007 Lee et al.
2008/0003142 A1 1/2008 Link et al.
2008/0004436 A1 1/2008 Tawfik et al.
2008/0014589 A1 1/2008 Link et al.
2008/0213766 A1 9/2008 Brown et al.
2008/0228268 A1 9/2008 Shannon et al.
2008/0241820 A1 10/2008 Krutzik et al.
2008/0242560 A1 10/2008 Gunderson et al.
2009/0005252 A1 1/2009 Drmanac et al.
2009/0011943 A1 1/2009 Drmanac et al.
2009/0012187 A1 1/2009 Chu et al.
2009/0025277 A1 1/2009 Takanashi
2009/0035770 A1 2/2009 Mathies et al.
2009/0048124 A1 2/2009 Leamon et al.
2009/0053169 A1 2/2009 Castillo et al.
2009/0068170 A1 3/2009 Weitz et al.
2009/0098555 A1 4/2009 Roth et al.
2009/0099041 A1 4/2009 Church et al.
2009/0105959 A1 4/2009 Braverman et al.
2009/0118488 A1 5/2009 Drmanac et al.
2009/0137404 A1 5/2009 Drmanac et al.
2009/0137414 A1 5/2009 Drmanac et al.
2009/0143244 A1 6/2009 Bridgham et al.
2009/0148961 A1 6/2009 Luchini et al.
2009/0155780 A1 6/2009 Xiao et al.
2009/0155781 A1 6/2009 Drmanac et al.
2009/0197248 A1 8/2009 Griffiths et al.
2009/0197772 A1 8/2009 Griffiths et al.
2009/0202984 A1 8/2009 Cantor
2009/0203531 A1 8/2009 Kurn
2009/0264299 A1 10/2009 Drmanac et al.
2009/0286687 A1 11/2009 Dressman et al.
2010/0021973 A1 1/2010 Makarov et al.
2010/0021984 A1 1/2010 Edd et al.
2010/0022414 A1 1/2010 Link et al.
2010/0035254 A1 2/2010 Williams
2010/0062494 A1 3/2010 Church et al.
2010/0069263 A1 3/2010 Shendure et al.
2010/0086914 A1 4/2010 Bentley et al.
2010/0105112 A1 4/2010 Holtze et al.
2010/0113296 A1 5/2010 Myerson
2010/0120098 A1 5/2010 Grunenwald et al.
2010/0130369 A1 5/2010 Shenderov et al.
2010/0136544 A1 6/2010 Agresti et al.
2010/0137163 A1 6/2010 Link et al.
2010/0173394 A1 7/2010 Colston, Jr. et al.
2010/0210479 A1 8/2010 Griffiths et al.
2010/0248237 A1 9/2010 Froehlich et al.
2011/0000560 A1 1/2011 Miller et al.
2011/0033548 A1 2/2011 Lai et al.
2011/0033854 A1 2/2011 Drmanac et al.
2011/0053798 A1 3/2011 Hindson et al.
2011/0059556 A1 3/2011 Strey et al.
2011/0071053 A1 3/2011 Drmanac et al.
2011/0086780 A1 4/2011 Colston, Jr. et al.
2011/0092376 A1 4/2011 Colston, Jr. et al.
2011/0092392 A1 4/2011 Colston, Jr. et al.
2011/0160078 A1 6/2011 Fodor et al.
2011/0195496 A1 8/2011 Muraguchi et al.
2011/0201526 A1 8/2011 Berka et al.
2011/0217736 A1 9/2011 Hindson
2011/0218123 A1 9/2011 Weitz et al.
2011/0263457 A1 10/2011 Krutzik et al.
2011/0267457 A1 11/2011 Weitz et al.
2011/0281738 A1 11/2011 Drmanac et al.
2011/0287435 A1 11/2011 Grunenwald et al.
2011/0305761 A1 12/2011 Shum et al.
2011/0319281 A1 12/2011 Drmanac
2012/0000777 A1 1/2012 Garrell et al.
2012/0010098 A1 1/2012 Griffiths et al.
2012/0010107 A1 1/2012 Griffiths et al.
2012/0015382 A1 1/2012 Weitz et al.
2012/0015822 A1 1/2012 Weitz et al.
2012/0071331 A1 3/2012 Casbon et al.
2012/0121481 A1 5/2012 Romanowsky et al.
2012/0132288 A1 5/2012 Weitz et al.
2012/0135893 A1 5/2012 Drmanac et al.
2012/0172259 A1 7/2012 Rigatti et al.
2012/0190032 A1 7/2012 Ness et al.
2012/0196288 A1 8/2012 Beer et al.
2012/0208724 A1 8/2012 Steemers et al.
2012/0211084 A1 8/2012 Weitz et al.
2012/0220494 A1 8/2012 Samuels et al.
2012/0220497 A1 8/2012 Jacobson et al.
2012/0222748 A1 9/2012 Weitz et al.
2012/0309002 A1 12/2012 Link
2012/0316074 A1 12/2012 Saxonov
2013/0018970 A1 1/2013 Woundy et al.
2013/0028812 A1 1/2013 Prieto et al.
2013/0046030 A1 2/2013 Rotem et al.
2013/0059310 A1 3/2013 Brenner et al.
2013/0078638 A1 3/2013 Berka et al.
2013/0079231 A1 3/2013 Pushkarev et al.
2013/0109575 A1 5/2013 Kleinschmidt et al.
2013/0130919 A1 5/2013 Chen et al.
2013/0157870 A1 6/2013 Pushkarev et al.
2013/0157899 A1 6/2013 Adler, Jr. et al.

(56) **References Cited**

U.S. PATENT DOCUMENTS

2013/0178368	A1	7/2013	Griffiths et al.
2013/0189700	A1	7/2013	So et al.
2013/0203675	A1	8/2013	Desimone et al.
2013/0210639	A1	8/2013	Link et al.
2013/0210991	A1	8/2013	Fonnum et al.
2013/0211055	A1	8/2013	Raines et al.
2013/0225418	A1	8/2013	Watson
2013/0274117	A1	10/2013	Church et al.
2013/0296173	A1	11/2013	Callow et al.
2013/0343317	A1	12/2013	Etemad et al.
2014/0057799	A1	2/2014	Johnson et al.
2014/0065234	A1	3/2014	Shum et al.
2014/0120529	A1	5/2014	Andersen et al.
2014/0155295	A1	6/2014	Hindson et al.
2014/0194323	A1	7/2014	Gillevet
2014/0199730	A1	7/2014	Agresti et al.
2014/0199731	A1	7/2014	Agresti et al.
2014/0206554	A1	7/2014	Hindson et al.
2014/0227684	A1	8/2014	Hindson et al.
2014/0227706	A1	8/2014	Kato et al.
2014/0228255	A1	8/2014	Hindson et al.
2014/0235506	A1	8/2014	Hindson et al.
2014/0287963	A1	9/2014	Hindson et al.
2014/0302503	A1	10/2014	Lowe et al.
2014/0357500	A1	12/2014	Vigneault et al.
2014/0378322	A1	12/2014	Hindson et al.
2014/0378345	A1	12/2014	Hindson et al.
2014/0378349	A1	12/2014	Hindson et al.
2014/0378350	A1	12/2014	Hindson et al.
2015/0005199	A1	1/2015	Hindson et al.
2015/0005200	A1	1/2015	Hindson et al.
2015/0011430	A1	1/2015	Saxonov
2015/0011432	A1	1/2015	Saxonov
2015/0072899	A1	3/2015	Ward et al.
2015/0111256	A1	4/2015	Church et al.
2015/0119280	A1	4/2015	Srinivas et al.
2015/0218633	A1	8/2015	Hindson et al.
2015/0224466	A1	8/2015	Hindson et al.
2015/0225777	A1	8/2015	Hindson et al.
2015/0225778	A1	8/2015	Hindson et al.
2015/0292988	A1	10/2015	Bharadwaj et al.
2015/0298091	A1	10/2015	Weitz et al.
2015/0299772	A1	10/2015	Zhang
2015/0353999	A1	12/2015	Agresti et al.
2015/0376605	A1	12/2015	Jarosz et al.
2015/0376609	A1	12/2015	Hindson et al.
2015/0376700	A1	12/2015	Schnall-Levin et al.
2015/0379196	A1	12/2015	Schnall-Levin et al.
2016/0024558	A1	1/2016	Hardenbol
2016/0032282	A1	2/2016	Vigneault et al.
2016/0122817	A1	5/2016	Jarosz et al.
2016/0231324	A1	8/2016	Zhao et al.
2016/0244742	A1	8/2016	Linnarsson et al.
2016/0244809	A1	8/2016	Belgrader et al.
2016/0257984	A1	9/2016	Hardenbol et al.
2016/0281160	A1	9/2016	Jarosz et al.
2016/0304860	A1	10/2016	Hindson et al.
2016/0348093	A1	12/2016	Price et al.
2017/0016041	A1	1/2017	Greenfield et al.
2017/0114390	A1	4/2017	Hindson et al.
2017/0145476	A1	5/2017	Ryvkin et al.
2017/0183701	A1	6/2017	Agresti et al.
2017/0260584	A1	9/2017	Zheng et al.

FOREIGN PATENT DOCUMENTS

EP	2145955	B1	2/2012
EP	1905828	B1	8/2012
EP	2136786	B1	10/2012
EP	1908832	B1	12/2012
EP	2540389	A1	1/2013
EP	2752664	A1	7/2014
GB	2097692	B	5/1985
GB	2485850	A	5/2012
JP	55949832	A	3/1984
JP	S60227826	A	11/1985
JP	2006507921	A	3/2006
JP	2006289250	A	10/2006
JP	2007015990	A	1/2007
JP	2007268350	A	10/2007
JP	2009208074	A	9/2009
WO	WO-8402000	A1	5/1984
WO	WO-9418218	A1	8/1994
WO	WO-9419101	A1	9/1994
WO	WO-9423699	A1	10/1994
WO	WO-9530782	A1	11/1995
WO	WO-9629629	A2	9/1996
WO	WO-9641011	A1	12/1996
WO	WO-9802237	A1	1/1998
WO	WO-9852691	A1	11/1998
WO	WO-9909217	A1	2/1999
WO	WO-9952708	A1	10/1999
WO	WO-0008212	A1	2/2000
WO	WO-0023181	A1	4/2000
WO	WO-0026412	A1	5/2000
WO	WO-0043766	A1	7/2000
WO	WO-0070095	A2	11/2000
WO	WO-0102850	A1	1/2001
WO	WO-0114589	A2	3/2001
WO	WO-0189787	A2	11/2001
WO	WO-0190418	A1	11/2001
WO	WO-0127610	A3	3/2002
WO	WO-0231203	A2	4/2002
WO	WO-02086148	A1	10/2002
WO	WO-0218949	A3	1/2003
WO	WO-03062462	A2	7/2003
WO	WO-2004002627	A2	1/2004
WO	WO-2004010106	A2	1/2004
WO	WO-2004061083	A2	7/2004
WO	WO-2004065617	A2	8/2004
WO	WO-2004069849	A2	8/2004
WO	WO-2004091763	A2	10/2004
WO	WO-2004102204	A1	11/2004
WO	WO-2004103565	A2	12/2004
WO	WO-2004105734	A1	12/2004
WO	WO-2005002730	A1	1/2005
WO	WO-2005021151	A1	3/2005
WO	WO-2005023331	A2	3/2005
WO	WO-2005040406	A1	5/2005
WO	WO-2005049787	A2	6/2005
WO	WO-2005082098	A2	9/2005
WO	WO-2006030993	A1	3/2006
WO	WO-2006078841	A1	7/2006
WO	WO-2006096571	A2	9/2006
WO	WO-2007001448	A2	1/2007
WO	WO-2007002490	A2	1/2007
WO	WO-2007018601	A1	2/2007
WO	WO-2007024840	A2	3/2007
WO	WO-2007081385	A2	7/2007
WO	WO-2007081387	A1	7/2007
WO	WO-2007089541	A2	8/2007
WO	WO-2007114794	A1	10/2007
WO	WO-2007121489	A2	10/2007
WO	WO-2007133710	A2	11/2007
WO	WO-2007138178	A2	12/2007
WO	WO-2007139766	A2	12/2007
WO	WO-2007140015	A2	12/2007
WO	WO-2007147079	A2	12/2007
WO	WO-2007149432	A2	12/2007
WO	WO-2008021123	A1	2/2008
WO	WO-2008091792	A2	7/2008
WO	WO-2008102057	A1	8/2008
WO	WO-2008109176	A2	9/2008
WO	WO-2008121342	A2	10/2008
WO	WO-2008061193	A3	11/2008
WO	WO-2008134153	A1	11/2008
WO	WO-2008150432	A1	12/2008
WO	WO-2009005680	A1	1/2009
WO	WO-2009011808	A1	1/2009
WO	WO-2009048532	A2	4/2009
WO	WO-2009061372	A1	5/2009
WO	WO-2009085215	A1	7/2009
WO	WO-2010004018	A2	1/2010
WO	WO-2010033200	A2	3/2010

(56) **References Cited**

FOREIGN PATENT DOCUMENTS

WO	WO-2010104604	A1	9/2010
WO	WO-2010115154	A1	10/2010
WO	WO-2010148039	A2	12/2010
WO	WO-2010151776	A2	12/2010
WO	WO-2010117620	A3	2/2011
WO	WO-2011028539	A1	3/2011
WO	WO-2011047870	A1	4/2011
WO	WO-2011056546	A1	5/2011
WO	WO-2011066476	A1	6/2011
WO	WO-2011074960	A1	6/2011
WO	WO-2011140627	A1	11/2011
WO	WO-2012012037	A1	1/2012
WO	WO-2012048341	A1	4/2012
WO	WO-2012083225	A2	6/2012
WO	WO-2012106546	A2	8/2012
WO	WO-2012112970	A2	8/2012
WO	WO-2012136734	A1	10/2012
WO	WO-2012142611	A2	10/2012
WO	WO-2012148497	A2	11/2012
WO	WO-2012149042	A2	11/2012
WO	WO-2012166425	A2	12/2012
WO	WO-2013019751	A1	2/2013
WO	WO-2013036929	A1	3/2013
WO	WO-2013122996	A1	8/2013
WO	WO-2013123125	A1	8/2013
WO	WO-2013126741	A1	8/2013
WO	WO-2013134261	A1	9/2013
WO	WO-2013150083	A1	10/2013
WO	WO-2013177220	A1	11/2013
WO	WO-2014028537	A1	2/2014
WO	WO-2014074611	A1	5/2014
WO	WO-2014093676	A1	6/2014
WO	WO-2014144495	A1	9/2014
WO	WO-2014210353	A2	12/2014
WO	WO-2015044428	A1	4/2015
WO	WO-2015164212	A1	10/2015
WO	WO-2016040476	A1	3/2016
WO	WO-2017053905	A1	3/2017
WO	WO-2017075265	A1	5/2017

OTHER PUBLICATIONS

Abate, et al. High-throughput injection with microfluidics using picoinjectors. Proc Natl Acad Sci U S A. Nov. 9, 2010;107(45):19163-6. doi: 10.1073/pnas.1006888107. Epub Oct. 20, 2010.

Abate et al., Valve-based flow focusing for drug formation. Appl Phys Lett. 2009;94. 3 pages.

Agresti, et al. Selection of ribozymes that catalyse multiple-turnover Diels-Alder cycloadditions by using in vitro compartmentalization. Proc Natl Acad Sci U S A. Nov. 8, 2005;102(45):16170-5. Epub Oct. 31, 2005.

Aitman, et al. Copy number polymorphism in Fcgr3 predisposes to glomerulonephritis in rats and humans. Nature. Feb. 16, 2006;439(7078):851-5.

Akselband. Enrichment of slow-growing marine microorganisms from mixed cultures using gel microdrop (GMD) growth assay and fluorescence-activated cell sorting. J. Exp. Marine Bioi., 329: 196-205 (2006).

Akselband. Rapid mycobacteria drug susceptibility testing using gel microdrop (GMD) growth assay and flow cytometry. J. Microbiol. Methods, 62:181-197 (2005).

Altemos et al. Genomic Characterization of Large Heterochromatic Gaps in the Human Genome Assembly. PLOS Computational Biology, May 15, 2014, vol. 10, Issue 5, 14 pages.

Amini, S. et al. Haplotype-resolved whole-genome sequencing by contiguity-preserving transposition and combinatorial indexing. Nature Genetics (2014) 46:1343-1349 doi:10.1038/ng.3119.

Anna, S.L., et al. Formation of dispersions using "flow focusing" in microchannels. Applied Physics Letters, vol. 82, No. 3, pp. 364-366 (2003).

Anonymous, "Oligo(dT)25 cellulose beads" NEB (2012) Retrieved from the Internet:https://www.neb.com/~/media/Catalog/All-Products/286CA51268E24DE1B06F1CB2886981354/Datacards%20or%Manuals/S1408Datasheet-Lot0011205.pdf.

Anonymous, "Oligotex Handbook" Qiagen (2012) XP055314680, Retrieved from the Internet: URL:http://www.qiagen.com/de/resources/download.apsx?id=f9fald98-d54d-47e7-a20b-8b0cb8975009&lang=en.

Anonymous: "Viscosity-Basic concepts" (2004) XP055314117, Retrieved from the Internet: URL:http://lhtc.epfl.ch/webdav/site/lhtc/shared/import/migration/2 VISCOSITY.pdf.

Attia, et al. Micro-injection moulding of polymer microfluidic devices. Microfluidics and Nanofluidics. 2009; 7(1):1-28.

Balikova, et al. Autosomal-dominant microtia linked to five tandem copies of a copy-number-variable region at chromosome 4p16. Am J Hum Genet. Jan. 2008;82(1):181-7. doi: 10.1016/j.ajhg.2007.08.001.

Berkum, et al. Hi-C: a method to study the three-dimensional architecture of genomes. J Vis Exp. May 6, 2010;(39). pii: 1869. doi: 10.3791/1869.

Biles et al., Nucl. Acids Res. 32(22):e176 2004.

Bodi, K. et al. Comparison of Commercially Available Target Enrichment Methods for Next-Generation Sequencing. J Biomolecular Techniques (2013) 24:73-86.

Boone, et al. Plastic advances microfluidic devices. The devices debuted in silicon and glass, but plastic fabrication may make them hugely successful in biotechnology application. Analytical Chemistry. Feb. 2002; 78A-86A.

Braeckmans et al. Scanning the Code. Modern Drug Discovery. 2003:28-32.

Bransky, et al. A microfluidic droplet generator based on a piezoelectric actuator. Lab Chip. Feb. 21, 2009;9(4):516-20. doi: 10.1039/b814810d. Epub Nov. 20, 2008.

Briggs, et al. Tumor-infiltrating immune repertoires captures by single-cell barcoding in emulsion with Supplementary material. bioRxiv 134841; doi: https://doi.org/10.1101/134841. Posted May 5, 2017.

Brouzes, et al. Droplet microfluidic technology for single-cell high-throughput screening. Proc Natl Acad Sci U S A. Aug. 25, 2009;106(34):14195-200. doi: 10.1073/pnas.0903542106. Epub Jul. 15, 2009.

Browning, et al. Haplotype phasing: existing methods and new developments. Nat Rev Genet. Sep. 16, 2011;12(10):703-14. doi: 10.1038/nrg3054. Review.

Cappuzzo, et al. Increased HER2 gene copy number is associated with response to gefitinib therapy in epidermal growth factor receptor-positive non-small-cell lung cancer patients. J Clin Oncol. Aug. 1, 2005;23(22):5007-18.

Carroll. The selection of high-producing cell lines using flow cytometry and cell sorting. Exp. Op. Biol. Therap., 4:11 1821-1829 (2004).

Chaudhary. A rapid method of cloning functional variable-region antibody genese in *Escherichia coli* as single-chain imrnunotoxins. Proc. Natl. Acad. Sci USA 87: 1066-1070 (Feb. 1990).

Chechetkin et al. Sequencing by hybridization with the generic 6-mer oligonucleotide microarray: an advanced scheme for data processing. J Biomol Struct Dyn. Aug. 2000;18(1):83-101.

Chen, et al. Chemical transfection of cells in picoliter aqueous droplets in fluorocarbon oil. Anal Chem. Nov. 15, 2011;83(22):8816-20. doi: 10.1021/ac2022794. Epub Oct. 17, 2011.

Choi, et al. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. Cancer Res. Jul. 1, 2008;68(13):4971-6. doi: 10.1158/0008-5472.CAN-07-6158.

Chokkalingam, et al. Probing cellular heterogeneity in cytokine-secreting immune cells using droplet-based microfluidics. Lab Chip. Dec. 21, 2013;13(24):4740-4. doi: 10.1039/c3lc50945a.

Chou, et al. Disposable Microdevices for DNA Analysis and Cell Sorting. Proc. Solid-State Sensor and Actuator Workshop, Hilton Head, SC. Jun. 8-11, 1998; 11-14.

Christian M, et al. Targeting DNA double-strand breaks with TAL effector nucleases. Genetics. 2010;186:757-761.

(56) **References Cited**

OTHER PUBLICATIONS

Christiansen et al. The Covalent Eukaryotic Topoisomerase I-DNA Intermediate Catalyzes pH-dependent Hydrolysis and Alcoholysis. J Biol Chem (Apr. 14, 1994) 269(15):11367-11373.
Chu, et al. Controllable monodisperse multiple emulsions. Angew Chem Int Ed Engl. 2007;46(47):8970-4.
Chung, et al. Structural and molecular interrogation of intact biological systems. Nature. May 16, 2013;497(7449):332-7. doi: 10.1038/nature12107. Epub Apr. 10, 2013.
Clausell-Tormos et al. Droplet-based microfluidic platforms for the encapsulation and screening of mammalian cells and multicellular organisms. Chem. Biol. 15:427-437 (2008).
Cong, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. Feb. 15, 2013;339(6121):819-23. doi: 10.1126/science.1231143. Epub Jan. 3, 2013.
Cook, et al. Copy-number variations associated with neuropsychiatric conditions. Nature. Oct. 16, 2008;455(7215):919-23. doi: 10.1038/nature07458.
Co-pending U.S. Appl. No. 15/355,542, filed Nov. 18, 2016.
Co-pending U.S. Appl. No. 15/392,557, filed Dec. 28, 2016.
Co-pending U.S. Appl. No. 15/430,298, filed Feb. 10, 2017.
Co-pending U.S. Appl. No. 15/440,772, filed Feb. 23, 2017.
Co-pending U.S. Appl. No. 15/449,741, filed Mar. 3, 2017.
Co-pending U.S. Appl. No. 15/470,814, filed Mar. 27, 2017.
Co-pending U.S. Appl. No. 15/588,519, filed May 5, 2017.
Coufal, et al. L1 retrotransposition in human neural progenitor cells. Nature. Aug. 27, 2009;460(7259):1127-31. doi: 10.1038/nature08248. Epub Aug. 5, 2009.
De Bruin et al., UBS Investment Research. Q-Series®: DNA Sequencing. UBS Securities LLC. Jul. 12, 2007. 15 pages.
Dekker, et al. Capturing chromosome conformation. Science. Feb. 15, 2002;295(5558):1306-11.
Demirci, et al. Single cell epitaxy by acoustic picolitre droplets. Lab Chip. Sep. 2007;7(9):1139-45. Epub Jul. 10, 2007.
Doerr. The smallest bioreactor. Nature Methods, 2:5 326 (2005).
Doshi, et al. Red blood cell-mimicking synthetic biomaterial particles. Proceedings of the National Academy of Sciences 106.51 (2009): 21495-21499.
Dowding, et al. Oil core/polymer shell microcapsules by internal phase separation from emulsion droplets. II: controlling the release profile of active molecules. Langmuir. Jun. 7, 2005;21(12):5278-84.
Draper, et al. Compartmentalization of electrophoretically separated analytes in a multiphase microfluidic platform. Anal Chem. Jul. 3, 2012;84(13):5801-8. doi: 10.1021/ac301141x. Epub Jun. 13, 2012.
Dressler, et al. Droplet-based microfluidics enabling impact on drug discovery. J Biomol Screen. Apr. 2014;19(4):483-96. doi: 10.1177/1087057113510401. Epub Nov. 15, 2013.
Dressman et al. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. Proc. Natl. Acad. Sci. 2003. 100(15):8817-8822.
Drmanac et al., Sequencing by hybridization (SBH): advantages, achievements, and opportunities. Adv Biochem Eng Biotechnol. 2002;77 :75-101.
Droplet Based Sequencing (slides) dated (Mar. 12, 2008).
Eastburn, et al. Ultrahigh-throughput mammalian single-cell reverse-transcriptase polymerase chain reaction in microfluidic droplets. Anal Chem. Aug. 20, 2013;85(16):8016-21. doi: 10.1021/ac402057q. Epub Aug. 8, 2013.
Esser-Kahn, et al. Triggered release from polymer capsules. Macromolecules. 2011; 44:5539-5553.
Fabi, et al. Correlation of efficacy between EGFR gene copy number and lapatinib/capecitabine therapy in HER2-positive metastatic breast cancer. J. Clin. Oncol. 2010; 28:15S. 2010 ASCO Meeting abstract Jun. 14, 2010:1059.
Fan, et al. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci U S A. Oct. 21, 2008;105(42):16266-71. doi: 10.1073/pnas.0808319105. Epub Oct. 6, 2008.
Fan, et al. Whole-genome molecular haplotyping of single cells. Nature Biotechnology, vol. 29, No. 1. Jan. 1, 2011. pp. 51-57.
Fang, et al. Fluoride-cleavable biotinylation phosphoramidite for 5'-end-labeling and affinity purification of synthetic oligonucleotides. Nucleic Acids Res. Jan. 15, 2003;31(2):708-15.
Fisher, et al. A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. Genome Biol. 2011;12(1):R1. doi: 10.1186/gb-2011-12-1-rl. Epub Jan. 4, 2011.
Frampton, G.M. et al. Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing. Nature Biotechnology (2013) 31(11):1023-1031. doi:10.1038/nbr.2696.
Fredrickson, et al. Macro-to-micro interfaces for microfluidic devices. Lab Chip. Dec. 2004;4(6):526-33. Epub Nov. 10, 2004.
Freiberg, et al. Polymer microspheres for controlled drug release. Int J Pharm. Sep. 10, 2004;282(1-2):1-18.
Fu, A.Y., et al. A microfabricated fluorescence-activated cell sorter. Nature Biotechnology, vol. 17, pp. 1109-1111 (1999).
Fulton et al., Advanced multiplexed analysis with the FlowMetrix system. Clin Chern. Sep. 1997;43(9): 1749-56.
Garstecki, et al. Formation of monodisperse bubbles in a microfluidic flow-focusing device. Applied Physics Letters. 2004; 85(13):2649-2651. DOI: 10.1063/1.1796526.
Gartner, et al. The Microfluidic Toolbox—examples for fluidic interfaces and standardization concepts. Proc. SPIE 4982, Microfluidics, BioMEMS, and Medical Microsystems, (Jan. 17, 2003); doi: 10.1117/12.479566.
Gericke, et al. Functional cellulose beads: preparation, characterization, and applications. Chemical reviews 113.7 (2013): 4812-4836.
Ghadessy, et al. Directed evolution of polymerase function by compartmentalized self-replication. Proc Natl Acad Sci U S A. Apr. 10, 2001;98(8):4552-7. Epub Mar. 27, 2001.
Gonzalez, et al. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science. Mar. 4, 2005;307(5714):1434-40. Epub Jan. 6, 2005.
Granieri, Lucia. Droplet-based microfluidics and engineering of tissue plasminogen activator for biomedical applications. Ph.D. Thesis, Nov. 13, 2009 (131 pages).
Grasland-Mongrain, et al. Droplet coalescence in microfluidic devices. Jan.-Jul. 2003. 31 pages. http://www.eleves.ens.fr/home/grasland/rapports/stage4.pdf.
Guo, et al. Droplet microfluidics for high-throughput biological assays. Lab Chip. Jun. 21, 2012;12(12):2146-55. doi: 10.1039/c21c21147e. Epub Feb. 9, 2012.
Gyarmati, et al. Reversible disulphide formation in polymer networks: a versatile functional group from synthesis to applications. European Polymer Journal. 2013; 49:1268-1286.
Hashimshony, et al. CEL-Seq: Single-Cell RNa-Seq by Multiplexed Linear Amplification. Cell Rep. Sep. 27, 2012;2(3):666-73. doi: 10.1016/j.celrep.2012.08.003. Epub Aug. 30, 2012.
He. Selective Encapsulation of Single Cells and Subcellular Organelles into Picoliter- and Femtoliter-Volume Droplets. Anal. Chem 77: 1539-1544 (2005).
He, J. et al. Genotyping-by-sequencing (GBS), an ultimate marker-assisted selections (MAS) tool to accelerate plant breeding. Frontiers in Plant Sci (Sep. 30, 2014) 5:1-8.
Hjerten, et al. General methods to render macroporous stationary phases nonporous and deformable, exemplified with agarose and silica beads and their use in high-performance ion-exchange and hydrophobic-interaction chromatography of proteins. Chromatographia 31.1-2 (1991): 85-94.
Holtze, et al. Biocompatible surfactants for water-in-fluorocarbon emulsions. Lab Chip. Oct. 2008;8(10):1632-9. doi: 10.1039/b806706f. Epub Sep. 2, 2008.
Huebner. Quantitative detection of protein expression in single cells using droplet microfluidics. Chem. Commun. 1218-1220 (2007).
Hug, et al. Measurement of the number of molecules of a single mRNA species in a complex mRNA preparation. J Theor Biol. Apr. 21, 2003;221(4):615-24.
Sigma. *Streptavidin-agarose* (S1638) product information sheet. www.sigma-aldrich.com.
Illumina Nextera Enrichment Sample Preparation Guide. Feb. 2013.
Illumina TruSeq Custom Enrichment Kit Data Sheet. (c) 2014.

(56) **References Cited**

OTHER PUBLICATIONS

Ioannidis, N. Manufacturing of Agarose-Based Chromatographic Adsorbents With Controlled Pore and Particle Sizes. A thesis submitted to the University of Birmingham for the degree of Doctor of Philosophy. 2009.

Jena, et al. Cyclic olefin copolymer based microfluidic devices for biochip applications: Ultraviolet surface grafting using 2-methacryloyloxyethyl phosphorylcholine. Biomicrofluidics. Mar. 2012;6(1):12822-1282212. doi: 10.1063/1.3682098. Epub Mar. 15, 2012.

Jung, et al. Micro machining of injection mold inserts for fluidic channel of polymeric biochips. Sensors. 2007; 7(8):1643-1654.

Kaper, et al. Supporting Information for "Whole-genome haplotyping by dilution, amplification, and sequencing." Proc Natl Acad Sci U S A. Apr. 2, 2013;110(14):5552-7. doi: 10.1073/pnas.1218696110. Epub Mar. 18, 2013.

Kaper, et al. Whole-genome haplotyping by dilution, amplification, and sequencing. Proc Natl Acad Sci U S A. Apr. 2, 2013;110(14):5552-7. doi: 10.1073/pnas.1218696110. Epub Mar. 18, 2013.

Karmakar, et al. Organocatalytic removal of formaldehyde adducts from RNA and DNA bases. Nat Chem. Sep. 2015;7(9):752-8. doi: 10.1038/nchem.2307. Epub Aug. 3, 2015.

Kebschull, et al. High-Throughput Mapping of Single-Neuron Projections by Sequencing of Barcoded RNA. Neuron. Sep. 7, 2016;91(5):975-87. doi: 10.1016/j.neuron.2016.07.036. Epub Aug. 18, 2016.

Khomiakov A et al. Analysis of perfect and mismatched DNA duplexes by a generic hexanucleotide microchip. Mol Bioi (Mosk). Jul.-Aug. 2003;37(4):726-41. Russian. Abstract only.

Kim et al., Albumin loaded microsphere of amphiphilic poly(ethylene glycol)/poly(a-ester) multiblock copolymer. Eu. J. Pharm. Sci. 2004;23:245-51. Available online Sep. 27, 2004.

Kim, et al. Fabrication of monodisperse gel shells and functional microgels in microfluidic devices. Angew Chem Int Ed Engl. 2007;46(11):1819-22.

Kim, et al. Rapid prototyping of microfluidic systems using a PDMS/polymer tape composite. Lab Chip. May 7, 2009;9(9):1290-3. doi: 10.1039/b818389a. Epub Feb. 10, 2009.

Kitzman, et al. Noninvasive whole-genome sequencing of a human fetus. Sci Transl Med. Jun. 6, 2012;4(137):137ra76. doi: 10.1126/scitranslmed.3004323.

Kivioj a, et al. Counting Absolute Numbers of Molecules Using Unique Molecular Identifiers. Nature Methods 9, 72-74 (2012).

Klein, et al. Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. Cell. May 21, 2015;161(5):1187-201. doi: 10.1016/j.cell.2015.04.044.

Knight, et al. Subtle chromosomal rearrangements in children with unexplained mental retardation. Lancet. Nov. 13, 1999;354(9191):1676-81.

Korlach et al., Methods in Enzymology, Real-Time DNA Sequencing from Single Polymerase Molecules, (2010) 472:431-455.

Koster et al. Drop-based microfluidic devices for encapsulation of single cells. Lab on a Chip The Royal Soc. of Chern. 8: 1110-1115 (2008).

Kutyavin, et al. Oligonucleotides containing 2-aminoadenine and 2-thiothymine act as selectively binding complementary agents. Biochemistry. Aug. 27, 1996;35(34):11170-6.

Lagus, et al. A review of the theory, methods and recent applications of high-throughput single-cell droplet microfluidics. J. Phys. D: Appl. Phys. (2013) 46:114005. (21 pages).

Lee, et al. ACT-PRESTO: Rapid and consistent tissue clearing and labeling method for 3-dimensional (3D) imaging. Sci Rep. Jan. 11, 2016;6:18631. doi: 10.1038/srep18631.

Lee; et al. Highly multiplexed subcellular RNA sequencing in situ. Science. Mar. 21, 2014;343(6177):1360-3. doi: 10.1126/science.1250212. Epub Feb. 27, 2014.

Lee, J-H. et al. Fluorescent in situ sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues. Nature Protocols (Feb. 12, 2015) 10(3):442-458.

Li, et al. A single-cell-based platform for copy number variation profiling through digital counting of amplified genomic DNA fragments. ACS Appl Mater Interfaces. Mar. 24, 2017. doi: 10.1021/acsami.7b03146. [Epub ahead of print].

Li, Y., et al. PEGylated PLGA Nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. Journal of Controlled Release, vol. 71, pp. 203-211 (2001).

Liu, et al. Preparation of uniform-sized PLA microcapsules by combining Shirasu porous glass membrane emulsification technique and multiple emulsion-solvent evaporation method. J Control Release. Mar. 2, 2005;103(1):31-43. Epub Dec. 21, 2004.

Liu, et al. Smart thermo-triggered squirting capsules for Nanoparticle delivery. Soft Matter. 2010; 6(16):3759-3763.

Loscertales, I.G., et al. Micro/Nano Encapsulation via Electrified Coaxial Liquid Jets. Science, vol. 295, pp. 1695-1698 (2002).

Love. A microengraving method for rapid selection of single cells producing antigen-specific antibodies. Nature Biotech, 24:6 703 (Jun. 2006).

Lowe, Adam J. Norbornenes and [n]polynorbornanes as molecular scaffolds for anion recognition. Ph.D. Thesis (May 2010). (361 pages).

Lupski. Genomic rearrangements and sporadic disease. Nat Genet. Jul. 2007;39(7 Suppl):S43-7.

Macosko, et al. Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. Cell. May 21, 2015;161(5):1202-14. doi: 10.1016/j.ce11.2015.05.002.

Mair, et al. Injection molded microfluidic chips featuring integrated interconnects. Lab Chip. Oct. 2006;6(10):1346-54. Epub Jul. 31, 2006.

Makino, et al. Preparation of hydrogel microcapsules: Effects of preparation conditions upon membrane properties. Colloids and Surfaces B: Biointerfaces. Nov. 1998; 12(2), 97-104.

Marcus. Gene method offers diagnostic hope. The Wall Street Journal. Jul. 11, 2012.

Matochko, et al. Uniform amplification of phage display libraries in monodisperse emulsions. Methods. Sep. 2012;58(1):18-27. doi: 10.1016/j.ymeth.2012.07.012. Epub Jul. 20, 2012.

Mazutis, et al. Selective droplet coalescence using microfluidic systems. Lab Chip. Apr. 24, 2012;12(10):1800-6. doi: 10.1039/c2lc40121e. Epub Mar. 27, 2012.

Merriman, et al. Progress in ion torrent semiconductor chip based sequencing. Electrophoresis. Dec. 2012;33(23):3397-417. doi: 10.1002/elps.201200424.

Microfluidic ChipShop. Microfluidic product catalogue. Mar. 2005.

Microfluidic ChipShop. Microfluidic product catalogue. Oct. 2009.

Miller JC, et al. An improved zinc-finger nuclease architecture for highly specific genome editing. Nat. Biotechnol. 2007;25:778-785.

Mirzabekov. DNA Sequencing by Hybridization—a Megasequencing Method and a Diagnostic Tool? Trends in Biotechnology 12(1): 27-32 (1994).

Moore, et al. Behavior of capillary valves in centrifugal microfluidic devices prepared by three-dimensional printing. Microfluidics and Nanofluidics. 2011; 10(4):877-888.

Morgan, et al. Chapter 12: Human microbiome analysis. PLoS Comput Biol. 2012;8(12):e1002808. doi: 10.1371/journal.pcbi.1002808. Epub Dec. 27, 2012.

Mouritzen et al. Single nucleotide polymorphism genotyping using locked nucleic acid (LNa). Expert Rev Mol Diagn. Jan. 2003;3(1):27-38.

Mozhanova, A.A. et al. Local elastic properties of biological materials studied by SFM (2003) XP055314108, Retrieved from the Internet: URL:http://www.ntmdt.com/data/media/files/publications/2003/08.08_a.a.mozhanova_n.i.n_english.pdf.

Muotri, et al. L1 retrotransposition in neurons is modulated by MeCP2. Nature. Nov. 18, 2010;468(7322):443-6. doi: 10.1038/nature09544.

Nagano, et al. Single-cell Hi-C reveals cell-to-cell variability in chromosome structure. Nature. Oct. 3, 2013;502(7469):59-64. doi: 10.1038/nature12593. Epub Sep. 25, 2013.

Nagashima, et al. Preparation of monodisperse poly (acrylamide-co-acrylic acid) hydrogel microspheres by a membrane emulsification technique and their size-dependent surface properties. Colloids and Surfaces B: Biointerfaces. Jun. 15, 1998; 11(1-2), 47-56.

(56) **References Cited**

OTHER PUBLICATIONS

Navin. The first five years of single-cell cancer genomics and beyond. Genome Res. Oct. 2015;25(10):1499-507. doi: 10.1101/gr.191098.115.

Nguyen, et al. In situ hybridization to chromosomes stabilized in gel microdrops. Cytometry. 1995; 21:111-119.

Novak, et al. Single cell multiplex gene detection and sequencing using microfluidicallygenerated agarose emulsions. Angew Chem Int Ed Engl. Jan. 10, 2011;50(2):390-5. doi: 10.1002/anie.201006089.

Oberholzer, et al. Polymerase chain reaction in liposomes. Chem Biol. Oct. 1995;2(10):677-82.

Ogawa, et al. Production and characterization of O/W emulsions containing cationic droplets stabilized by lecithin-chitosan membranes. J Agric Food Chem. Apr. 23, 2003;51(9):2806-12.

Okushima, S., et al. Controlled Production of Monodisperse Double Emulsions by Two-Step Droplet Breakup in Microfluidic Devices. Langmuir, vol. 20, pp. 9905-9908 (2004).

Oligotex Handbook. For purification of poly A+ RNA from total RNA and directly from cultured cells or tissues as well as purification of polyadenylated in vitro transcripts. Jun. 2012.

Pantel, et al. Detection methods of circulating tumor cells. J Thorac Dis. Oct. 2012;4(5):446-7. doi: 10.3978/j.issn.2072-1439.2012.08.15.

Patel, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. Science. Jun. 20, 2014;344(6190):1396-401. doi: 10.1126/science.1254257. Epub Jun. 12, 2014.

Perez, C., et al. Poly(lactic acid)-poly(ethylene glycol) Nanoparticles as new carriers for the delivery ofplasmid DNa. Journal of Controlled Release, vol. 75, pp. 211-224 (2001).

Peters, et al. Accurate whole-genome sequencing and haplotyping from 10 to 20 human cells. Nature. Jul. 11, 2012;487(7406):190-5. doi: 10.1038/Nature11236.

Picot, J. et al. A biomimetic microfluidic chip to study the circulation and mechanical retention of red blood cells in the spleen. Am J Hematology (Jan. 12, 2015) 90(4):339-345.

Pinto, et al. Functional impact of global rare copy number variation in autism spectrum disorders. Nature. Jul. 15, 2010;466(7304):368-72. doi: 10.1038/nature09146. Epub Jun. 9, 2010.

Plunkett, et al. Chymotrypsin responsive hydrogel: application of a disulfide exchange protocol for the preparation of methacrylamide containing peptides. Biomacromolecules. Mar.-Apr. 2005;6(2):632-7.

Porteus MH, Baltimore D. Chimeric nucleases stimulate gene targeting in human cells. Science. 2003;300:763.

Ram, et al. Strategy for microbiome analysis using 16S rRNA gene sequence analysis on the Illumina sequencing platform. Syst Biol Reprod Med. Jun. 2011;57(3):162-70. doi: 10.3109/19396368.2011.555598. Epub Mar. 1, 2011.

Ran, et al., Genome Engineering Using the CRISPR-Cas9 System, Nature Protocol, (2013), 8(11):2281-2308.

Richardson, et al. Novel inhibition of archaeal family-D DNA polymerase by uracil. Nucleic acids research 41.7 (2013): 4207-4218.

Rogozin, et al. A highly conserved family of inactivated archaeal B family DNA polymerases. Biol Direct. Aug. 6, 2008;3:32. doi: 10.1186/1745-6150-3-32.

Ropers. New perspectives for the elucidation of genetic disorders. Am J Hum Genet. Aug. 2007;81(2):199-207. Epub Jun. 29, 2007.

Rotem, et al. High-Throughput Single-Cell Labeling (Hi-SCL) for RNA-Seq Using Drop-Based Microfluidics. PLoS One. May 22, 2015;10(5):e0116328. doi: 10.1371/journal.pone.0116328. eCollection 2015.

Rotem, et al. Single Cell Chip-Seq Using Drop-Based Microfluidics. Abstract #50. Frontiers of Single Cell Analysis, Stanford University Sep. 5-7, 2013.

Rotem, et al. Single-cell ChIP-seq reveals cell subpopulations defined by chromatin state. Nat Biotechnol. Nov. 2015;33(11):1165-72. doi: 10.1038/nbt.3383. Epub Oct. 12, 2015.

Ryan. Rapid assay for mycobacterial growth and antibiotic susceptibility using gel microdrop and encapsulation. J. Clinical Microbial., 33:7 1720-1726 (1995).

Sander JD, et al. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). Nat. Methods. 2011;8:67-69.

Schirinzi et al., Combinatorial sequencing-by-hybridization: aNalysis of the NFI gene. Genet Test. 2006 Spring;10(1):8-17.

Schmeider, et al. Fast identification and removal of sequence contamination from genomic and metagenomic datasets. PLoS One. Mar. 9, 2011;6(3):e17288. doi: 10.1371/journal.pone.0017288.

Schmitt. Bead-based multiplex genotyping of human papillomaviruses. J. Clinical Microbial., 44:2 504-512 (2006).

Sebat, et al. Strong association of de novo copy number mutations with autism. Science. Apr. 20, 2007;316(5823):445-9. Epub Mar. 15, 2007.

Seiffert, et al. Smart microgel capsules from macromolecular precursors. J Am Chem Soc. May 12, 2010;132(18):6606-9. doi: 10.1021/ja102156h.

Shah. Fabrication of mono disperse thermosensitive microgels and gel capsules in micro fluidic devices. Soft Matter, 4:2303-2309 (2008).

Shimkus, et al. A chemically cleavable biotinylated nucleotide: usefulness in the recovery of protein-DNA complexes from avidin affinity columns. Proc Natl Acad Sci U S A. May 1985;82(9):2593-7.

Shlien, et al. Copy number variations and cancer. Genome Med. Jun. 16, 2009;1(6):62. doi: 10.1186/gm62.

Shlien, et al. Excessive genomic DNA copy number variation in the Li-Fraumeni cancer predisposition syndrome. Proc Natl Acad Sci U S A. Aug. 12, 2008;105(32):11264-9. doi: 10.1073/pnas.0802970105. Epub Aug. 6, 2008.

Shuttleworth, et al. Recognition of the pro-mutagenic base uracil by family B DNA polymerases from archaea. J Mol Biol. Mar. 26, 2004;337(3):621-34.

Simeonov et al., Single nucleotide polymorphism genotyping using short, fluorescently labeled locked nucleic acid (LNa) probes and fluorescence polarization detection. Nucleic Acids Res. Sep. 1, 2002;30(17):e91.

Sorokin et al., DiscrimiNation between perfect and mismatched duplexes with oligonucleotide gel microchips: role of thermodynamic and kinetic effects during hybridization. J Biomol Struct Dyn. Jun. 2005;22(6):725-34.

Su, et al., Microfluidics-Based Biochips: Technology Issues, Implementation Platforms, and Design-Automation Challenges. IEEE Transactions on Computer-Aided Design of Integrated Circuits and Systems. 2006;25(2):211-23. (Feb. 2006).

Sun et al., Progress in research and application of liquid-phase chip technology. Chinese JourNal Experimental Surgery. May 2005;22(5):639-40.

Susaki, et al. Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. Cell. Apr. 24, 2014;157(3):726-39. doi: 10.1016/j.ce11.2014.03.042. Epub Apr. 17, 2014.

Tawfik, D.S., et al. Man-made cell-like compartments for molecular evolution. Nature Biotechnology, vol. 16, pp. 652-656 (1998).

Tayyab, S. et al. Size exclusion chromatography and size exclusion HPLC of proteins Biochem Ed, Pergamon, (1991) 19(3):149-152.

Tewhey, et al. Microdroplet-based PCR enrichment for large-scale targeted sequencing. Nat Biotechnol. Nov. 2009;27(11):1025-31. doi: 10.1038/nbt.1583. Epub Nov. 1, 2009.

Theberge, et al. Microdropelts in microfluidics: an evolving platform for discoveries in chemsitry and biology. Angew Chem Int Ed Engl. Aug. 9, 2010;49(34):5846-68. doi: 10.1002/anie.200906653.

Tomer, et al. Advanced CLARITY for rapid and high-resolution imaging of intact tissues. Nat Protoc. Jul. 2014;9(7):1682-97. doi: 10.1038/nprot.2014.123. Epub Jun. 19, 2014.

Tonelli, et al. Perfluoropolyether functional oligomers: unusual reactivity in organic chemistry. Journal of fluorine chemistry. 2002; 118(1)"107-121.

(56) **References Cited**

OTHER PUBLICATIONS

Tubeleviciute, et al. Compartmentalized self-replication (CSR) selection of Thermococcus litoralis ShlB DNa polymerase for diminished uracil binding. Protein Eng Des Sel. Aug. 2010;23(8):589-97. doi: 10.1093/protein/gzq032. Epub May 31, 2010.

Turner, et al. Methods for genomic partitioning. Annu Rev Genomics Hum Genet. 2009;10:263-84. doi: 10.1146/annurev-genom-082908-150112. Review.

Wagner, et al. Biocompatible fluorinated polyglycerols for droplet microfluidics as an alternative to PEG-based copolymer surfactants. Lab Chip. Jan. 7, 2016;16(1):65-9. doi: 10.1039/c5lc00823a. Epub Dec. 2, 2015.

Wang, et al. A novel thermo-induced self-bursting microcapsule with magnetic-targeting property. Chemphyschem. Oct. 5, 2009;10(14):2405-9.

Wang, et al. Digital karyotyping. Proc Natl Acad Sci U S A. Dec. 10, 2002;99(25):16156-61. Epub Dec. 2, 2002.

Wang et al., Single nucleotide polymorphism discrimination assisted by improved base stacking hybridization using oligonucleotide microarrays. Biotechniques. 2003;35:300-08.

Weaver. Rapid clonal growth measurements at the single-cell level: gel microdroplets and flow cytometry. Biotechnology, 9:873-877 (1991).

Wesolowska, et al. Cost-effective multiplexing before capture allows screening of 25 000 clinically relevant SNPs in childhood acute lymphoblastic leukemia. Leukemia. Jun. 2011;25(6):1001-6. doi: 10.1038/leu.2011.32. Epub Mar. 18, 2011.

Whitesides. Soft lithography in biology and biochemistry. Annual Review of Biomedical Engineering, 3:335-373 (2001).

Williams et al., Amplification of complex gene libraries by emulsion PCR, Nature Methods 3(7):545-550 (2006).

Woo, et al. G/C-modified oligodeoxynucleotides with selective complementarity: synthesis and hybridization properties. Nucleic Acids Res. Jul. 1, 1996;24(13):2470-5.

Wood AJ, et al. Targeted genome editing across species using ZFNs and TALENs. Science. 2011;333:307.

Xia and Whitesides, Soft Lithography, Ann. Rev. Mat. Sci. 28:153-184 (1998).

Yamamoto, et al. Chemical modification of Ce(IV)/EDTA-base artificial restriction DNA cutter for versatile manipulation of double-stranded DNA. Nucleic Acids Research. 2007; 35(7):e53.

Zhang. Combinatorial marking of cells and organelles with reconstituted fluorescent proteins. Cell, 119:137-144 (Oct. 1, 2004).

Zhang, et al. Degradable disulfide core-cross-linked micelles as a drug delivery system prepared from vinyl functionalized nucleosides via the RAFT process. Biomacromolecules. Nov. 2008;9(11):3321-31. doi: 10.1021/bm800867n. Epub Oct. 9, 2008.

Zhang F, et al. Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. Nat. Biotechnol. 2011;29:149-153.

Zhao, J., et al. Preparation of hemoglobin-loaded Nano-sized particles with porous structure as oxygen carriers. Biomaterials, vol. 28, pp. 1414-1422 (2007).

Zheng, et al. Massively parallel digital transcriptional profiling of single cells. Nat Commun. Jan. 16, 2007;8:14049. doi: 10.1038/ncomms14049.

Zheng, X.Y. et al. Haplotyping germline and cancer genomes with high-throughput linked-read sequencing. Nature Biotech (Feb. 1, 2016) 34(3):303-311.

Zhu et al. Hydrogel Droplet Microfluidics for High-Throughput Single Molecule/Cell Analysis. Accounts of Chemical Research Article ASAP. DOI: 10.1021/acs.accounts.6b00370.

Zhu, et al. Synthesis and self-assembly of highly incompatible polybutadienepoly(hexafluoropropoylene oxide) diblock copolymers. Journal of Polymer Science Part B: Polymer Physics. 2005; 43(24):3685-3694.

Zimmermann et at., Microscale production of hybridomas by hypo-osmolar electrofusion. Hum• Antibodies Hybridomas. Jan. 1992; 3(1): 14-8.

Zong, et al. Genome-wide detection of single-nucleotide and copy-number variations of a single human cell. Science. Dec. 21, 2012;338(6114):1622-6. doi: 10.1126/science.1229164.

Ahern, "Biochemical, Reagents Kits Offer Scientists Good Return on Investment" The Scientist (1995) 9(15):1-7.

Bentzen, et al. Large-scale detection of antigen-specific T cells using peptide-MHC-I multimers labeled with DNA barcodes. Nat Biotechnol. Oct. 2016;34(10):1037-1045. doi: 10.1038/nbt.3662. Epub Aug. 29, 2016.

Boulanger, et al, "Massively parallel haplotyping on microscopic beads for the high-throughput phase analysis of single molecules", PLoS One, vol. 7:1-10, 2012.

Brown, K., Targeted Sequencing Using Droplet-Based Microfluidics, RainDance Technologies, 2009, 1-18.

Casbon, et al, "Reflex: intramolecular barcoding of long-range PCR products for sequencing multiple pooled DNAs", Nucleic Acids Res., pp. 1-6, 2013.

Co-pending U.S. Appl. No. 15/596,754, filed May 16, 2017.

Co-pending U.S. Appl. No. 15/598,898, filed May 18, 2017.

Hiatt, et al. Parallel, tag-directed assembly of locally derived short sequence reads. Nat Methods. Feb. 2010;7(2):119-22. doi: 10.1038/nmeth.1416. Epub Jan. 17, 2010.

Hirsch et al. (2002) "Easily reversible desthiobiotin binding to streptavidin, avidin, and other biotin-binding proteins: uses for protein labeling, detection, and isolation." Analytical of Biochemistry 308(2):343-357.

Imburgio, et al, "Studies of promoter recognition and start site selection by T7 RNA polymerase using a comprehensive collection of promoter variants", Biochemistry., 39:10419-30, 2000.

Kirkness et al. "Sequencing of isolated sperm cells for direct haplotyping of a human genome," Genome Res (2013) 23:826-832.

Kitzman et al. Haplotype-resolved genome sequencing of a Gujarati Indian individual.â€ Nat Biotechnol (2011) 29:59-63.

Konarewa, et al, "96-plex molecular barcoding for the Illumina Genome Analyzer", Methods Mol Biol., 733:279-98, 2011. cited by applicant.

Kozarewa, et al. "Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of GC-biased genomes", Nat Methods., 6: 291-5, 2009. cited by applicant.

Kwok, et al, "Single-molecule analysis for molecular haplotyping", Hum Mutat., 23:442-6, 2004. cited by applicant.

Laird et al, Hairpin-bisulfite PCR: Assessing epigenetic methylation patterns on complementary strands of individual DNA molecules, 2004, PNAS, 101, 204-209.

Lan, et al. "Single-cell genome sequencing at ultra-high-throughput with microfluidic droplet barcoding" with Supplementary Material. Nat Biotechnol. May 29, 2017. doi: 10.1038/nbt.3880. [Epub ahead of print].

Linch, et al. Bone marrow processing and cryopreservation. Journal of Clinical Pathology; Feb. 1982, vol. 35, No. 2; pp. 186-190.

Lundin, et al, "Hierarchical molecular tagging to resolve long continuous sequences by massively parallel sequencing", Sci Rep., 3:1186, 2003.

Myllykangas et al., Targeted Sequencing Library Preparation by Genomic DNA Circularization, BMC Biotechnology, 2011, 11(122), 1-12.

Oyola, et al, "Optimizing Illumina next-generation sequencing library preparation for extremely AT-biased genomes", BMC Genomics.,13:1, 2012.

PCT/IB2010/002243, International Search Report and Written Opinion, dated Feb. 9, 2011, 13pgs.

Ramskold et al. (2012) "Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells" Nature Biotechnology 30(8):777-782.

Reisner, et al, "Single-molecule denaturation mapping of DNA in nanofluidic channels", Proc Natl Acad Sci U.S.A., 107: 13294-9, 2010.

Repp et al. "Genotyping by Multiplex Polymerase Chain Reaction for Detection of Endemic Hepatitis B Virus Transmission" J Clinical Microbiology (1993) 31:1095-1102.

(56) **References Cited**

OTHER PUBLICATIONS

Roche. Using Multiplex Identifier (MID) Adaptors for the GS FLX Titanium Chemistry Basic Mid Set Genome Sequencer FLX System, Technical Bulletin 004-2009, (Apr. 1, 2009) pp. 1-7. URL:http://454.com/downloads/my454/documentation/technical-bulletins/TCB-09004 Using MultiplexIdentifierAdaptors ForTheGSFLXTitaniumSeriesChemistry-BasicMIDSet.pdf.

Roche. Using Multiplex Identifier (MID) Adaptors for the GS FLX Titanium Chemistry Extended MID Set Genome Sequencer FLX System, Technical Bulletin 005-2009, (Apr. 1, 2009) pp. 1-7. URL:http://454.com/downloads/my454/documentation/technical-bulletins/TCB-09005 UsingMultiplexIdentifierAdaptors ForTheGSFLXTitaniumChemistry-ExtendedMIDSet.pdf.

Schwartz; et al., "Capturing native long-range contiguity by in situ library construction and optical sequencing", PNAS (Nov. 2012), 109(46)18749-18754.

Stoeckius, et al. Large-scale simultaneous measurement of epitopes and transcriptomes in single cells. bioRxiv 113068; doi: https://doi.org/10.1101/113068.

Tewhey et al., Supplementary Materials, Nature Biotechnology, 2009, 27(11), 1-22.

Turner, et al. Assaying chromosomal inversions by single-molecule haplotyping. Nat Methods. Jun. 2006;3(6):439-45.

Turner, et al, "High-throughput haplotype determination over long distances by haplotype fusion PCR and ligation haplotyping", Nat Protoc., 4:1771-83, 2009.

Ushijima et al, Detection and interpretation of altered methylation patterns in cancer cells, 2005, Nature reviews, 5, 223-231.

Van Nieuwerburgh, et al, "Illumina mate-paired DNA sequencing-library preparation using Cre-Lox recombination", Nucleic Acids Res., 40:1-8, 2012.

Wang; et al., "Self-Formed Adaptor PCR: a Simple and Efficient Method for Chromosome Walking", Applied and Environmental Microbiology (Aug. 2007), 73(15):5048-5051.

Wiseman, R.W. et al. "Major histocompatibility complex genotyping with massively parallel pyrosequencing" Nature Medicine (Oct. 11, 2009) 15(11):1322-1326.

Xiao, et al, "Determination of haplotypes from single DNA molecules: a method for single-molecule barcoding", Hum Mutat., 28:913-21, 2007.

Yan, Pu et al. "Rapid one-step construction of hairpin RNA" Biochem and Biophys Res Comm (Jun. 12, 2009) 383(4):464-468.

Zhang. Genomics of inherited bone marrow failure and myelodysplasia. Dissertation [online]. University of Washington. 2015 [Retrieved on May 3, 2017].

Burns, et al. An Integrated Nanoliter DNA Analysis Device. Science. Oct. 16, 1998;282(5388):484-7.

Burns, et al. Microfabricated structures for integrated DNA analysis. Proc Natl Acad Sci U S A. May 28, 1996; 93(11): 5556-5561.

Burns, et al. The intensification of rapid reactions in multiphase systems using slug flow in capillaries. Lab Chip. Sep. 2001;1(1):10-5. Epub Aug. 9, 2001.

Curcio. Improved Techniques for High-Throughput Molecular Diagnostics. PhD Thesis. 2002.

Damean, et al. Simultaneous measurement of reactions in microdroplets filled by concentration gradients. Lab Chip. Jun. 21, 2009;9(12):1707-13. doi: 10.1039/b821021g. Epub Mar. 19, 2009.

Han, X. et al. "CRISPR-Cas9 delivery to hard-to-transfect cells via membrane deformation" Science Advances (2015) 1(7): E1500454 (8 pages).

Hosokawa, et al. Massively parallel whole genome amplification for single-cell sequencing using droplet microfluidics. Scientific Reports 7, Article No. 5199 (2017).

Katsura, et al. Indirect micromanipulation of single molecules in water-in-oil emulsion. Electrophoresis. Jan. 2001;22(2):289-93.

Kenis, et al. Microfabrication Inside Capillaries Using Multiphase Laminar Flow Patterning. Science. Jul. 2, 1999;285(5424):83-5.

Lagally, et al. Single-Molecular DNA Amplification and Analysis in an Integrated Microfluidic Device. Anal Chem. Feb. 1, 2001;73(3):565-70.

Man. Monolithic Structures for Integrated Microfluidic Analysis. PhD Thesis. 2001.

Nisisako, et al. Droplet formation in a microchannel network. Lab Chip. Feb. 2002;2(1):24-6. Epub Jan. 18, 2002.

Nisisako, T. et al. "Droplet Formation in a Microchannel on PMMA Plate" Abstract. 2001 Kluwer Academic Publishers. p. 137-138.

Rakszewska, A. et al. "One drop at a time: toward droplet microfluidics as a versatile tool for single-cell analysis" NPG Asia Materials (2014) 6(10):e133 (12 pages).

Ramsey, J.M. "The burgeoning power of the shrinking laboratory" Nature Biotech (1999) 17:1061-1062.

Reis, A. et al. "CRISPR/Cas9 and Targeted Genome Editing: A New Era in Molecular Biology" (2014) XP002766825: URL:https://www.neb.com/tools-and-resources/feabture-articles/crispr-cas9-and-targeted-genome-editing-a-new-era-in-molecular-biology.

Rodrigue, S. et al. "Whole genome amplification and de novo assembly of single bacterial cells" PLoS One. Sep. 2, 2009;4(9):e6864. doi: 10.1371/journal.pone.0006864.

Schubert, et al. Microemulsifying fluorinated oils with mixtures of fluorinated and hydrogenated surfactants. Colloids and Surfaces A; Physicochemical and Engineering Aspects, 84(1994) 97-106.

Song, et al. Reactions in droplets in microfluidic channels. Angew Chem Int Ed Engl. Nov. 13, 2006;45(44):7336-56.

Thorsen, et al. Dynamic pattern formation in a vesicle-generating microfluidic device. Physical Review Letters. American Physical Society. 2001; 86(18):4163-4166.

Ward, et al. Microfluidic flow focusing: Drop size and scaling in pressure versus flow-rate-driven pumping. Electrophoresis. Oct. 2005;26(19):3716-24.

Weigl, et al. Microfluidic Diffusion-Based Separation and Detection. Science. 1999; pp. 346-347.

Zeng, et al. High-performance single cell genetic analysis using microfluidic emulsion generator arrays. Anal Chem. Apr. 15, 2010;82(8):3183-90. doi: 10.1021/ac902683t.

Xi, et al. New library construction method for single-cell genomes. PLoS One. Jul. 19, 2017;12(7):e0181163. doi: 10.1371/journal.pone.0181163. eCollection 2017.

Baret, et al. Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. Lab Chip. Jul. 7, 2009;9(13):1850-8. doi: 10.1039/b902504a. Epub Apr. 23, 2009.

BD. BD Rhapsody™ Single-Cell Analysis System: Analyze hundreds of genes across tens of thousands of single cells in parallel. BD, Becton, Dickinson and Company. BDGM1012 Rev. 1. 2017. 8 pages.

Buchman GW, et al. Selective RNA amplification: a novel method using dUMP-containing primers and uracil DNA glycosylase. PCR Methods Appl. 1993 Aug; 3(1):28-31.

Co-pending U.S. Appl. No. 15/687,357, filed Aug. 25, 2017.

Co-pending U.S. Appl. No. 15/687,856, filed Aug. 28, 2017.

Co-pending U.S. Appl. No. 15/693,374, filed Aug. 31, 2017.

Co-pending U.S. Appl. No. 15/717,840, filed Sep. 27, 2017.

Co-pending U.S. Appl. No. 15/717,847, filed Sep. 27, 2017.

Co-pending U.S. Appl. No. 15/717,871, filed Sep. 27, 2017.

Co-pending U.S. Appl. No. 15/718,764, filed Sep. 28, 2017.

Co-pending U.S. Appl. No. 15/718,893, filed Sep. 28, 2017.

Co-pending U.S. Appl. No. 15/719,459, filed Sep. 28, 2017.

Co-pending U.S. Appl. No. 15/720,085, filed Sep. 29, 2017.

Dressman et al. Supplementary Information pp. 1-2 of article published 2003, PNAS 100(15:8817-22).

Hosono S, et al. Unbiased whole-genome amplification directly from clinical samples. Genome Res. May 2003; 13(5):954-64. Epub Apr. 14, 2003.

Kamperman, et al. Centering Single Cells in Microgels via Delayed Crosslinking Supports Long-Term 3D Culture by Preventing Cell Escape. Small. Jun. 13, 2017(22). doi: 10.1002/smll.201603711. Epub Apr. 28, 2017.

Lienemann, et al. Single cell-laden protease-sensitive microniches for long-term culture in 3D. Lab Chip. Feb. 14, 2017;17(4):727-737. doi: 10.1039/c6lc01444e.

Lo, et al. On the design of clone-based haplotyping. Genome Biol. 2013;14(9):R100.

(56) **References Cited**

OTHER PUBLICATIONS

Maricic T, et al. Optimization of 454 sequencing library preparation from small amounts of DNA permits sequence determination of both DNA strands. Biotechniques. Jan. 2009; 46(1):51-2, 54-7.
Perrott, Jimmy. Optimization and Improvement of Emulsion PCR for the Ion Torrent Next-Generation Sequencing Platform. (2011) Thesis.
Shahi, et al. Abseq: Ultrahigh-throughput single cell protein profiling with droplet microfluidic barcoding. Sci Rep. 2017; 7: 44447. Published online Mar. 14, 2017. doi: 10.1038/srep44447.
Skerra A. Phosphorothioate primers improve the amplification of DNA sequences by DNA polymerases with proofreading activity. Nucleic Acids Res. Jul. 25, 1992; 20(14):3551-4.
Stoeckius, et al. Simultaneous epitope and transcriptome measurement in single cells. Nature methods. Jul. 31, 2017. Supplemental Materials.

* cited by examiner

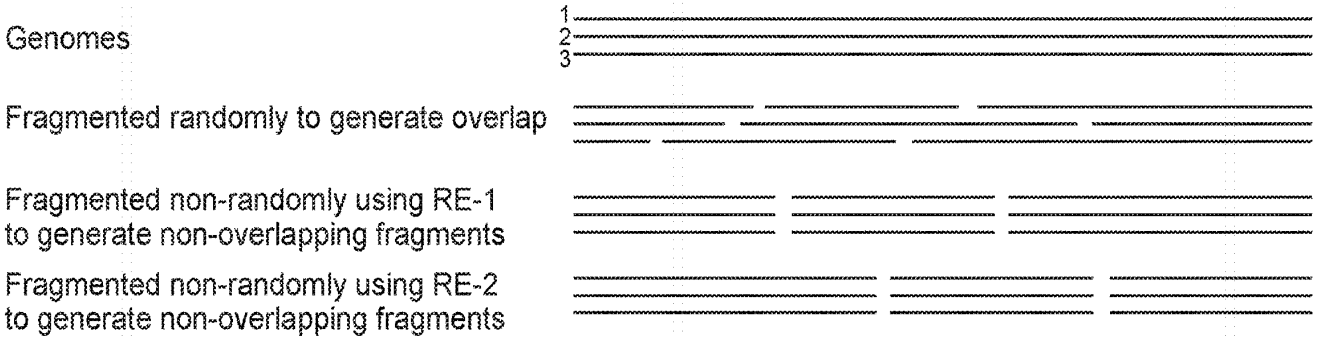
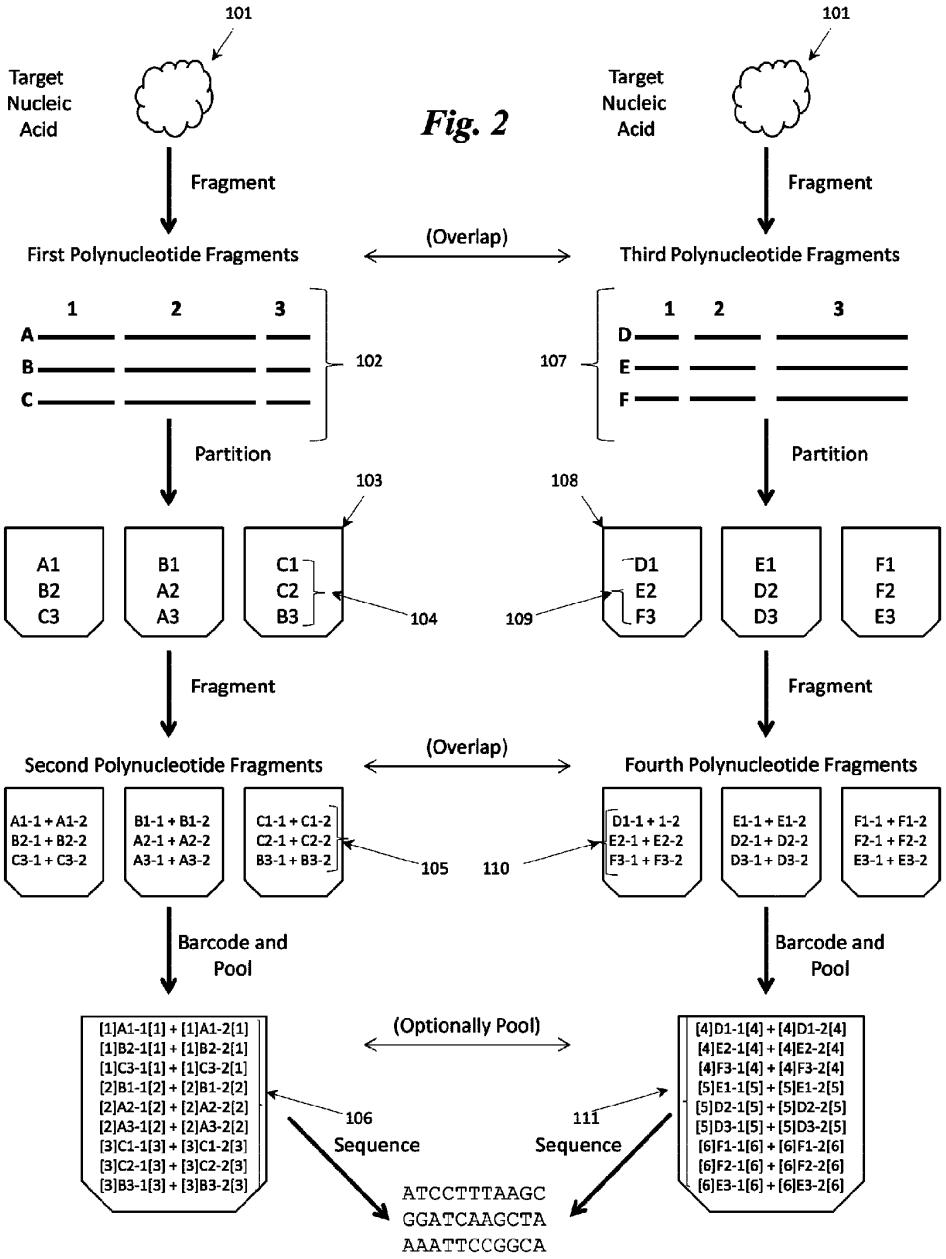

Genomes
1
2
3
Fragmented randomly to generate overlap
Fragmented non-randomly using RE-1
to generate non-overlapping fragments
Fragmented non-randomly using RE-2
to generate non-overlapping fragments

FIG. 1

Fig. 2


101
Target Nucleic Acid
Fragment
First Polynucleotide Fragments
(Overlap)
Third Polynucleotide Fragments
1 2 3
A
B
C
102
D
E
F
107
Partition
103
108
A1 B2 C3
B1 A2 A3
C1 C2 B3
104
D1 E2 F3
109
E1 D2 D3
F1 F2 E3
Second Polynucleotide Fragments
Fourth Polynucleotide Fragments
A1-1 + A1-2
B2-1 + B2-2
C3-1 + C3-2
B1-1 + B1-2
A2-1 + A2-2
A3-1 + A3-2
C1-1 + C1-2
C2-1 + C2-2
B3-1 + B3-2
105
110
D1-1 + 1-2
E2-1 + E2-2
F3-1 + F3-2
E1-1 + E1-2
D2-1 + D2-2
D3-1 + D3-2
F1-1 + F1-2
F2-1 + F2-2
E3-1 + E3-2
Barcode and Pool
(Optionally Pool)
[1]A1-1[1] + [1]A1-2[1]
[1]B2-1[1] + [1]B2-2[1]
[1]C3-1[1] + [1]C3-2[1]
[2]B1-1[2] + [2]B1-2[2]
[2]A2-1[2] + [2]A2-2[2]
[2]A3-1[2] + [2]A3-2[2]
[3]C1-1[3] + [3]C1-2[3]
[3]C2-1[3] + [3]C2-2[3]
[3]B3-1[3] + [3]B3-2[3]
106
Sequence
111
Sequence
[4]D1-1[4] + [4]D1-2[4]
[4]E2-1[4] + [4]E2-2[4]
[4]F3-1[4] + [4]F3-2[4]
[5]E1-1[5] + [5]E1-2[5]
[5]D2-1[5] + [5]D2-2[5]
[5]D3-1[5] + [5]D3-2[5]
[6]F1-1[6] + [6]F1-2[6]
[6]F2-1[6] + [6]F2-2[6]
[6]E3-1[6] + [6]E3-2[6]
ATCCTTTAAGC
GGATCAAGCTA
AAATTCCGGCA

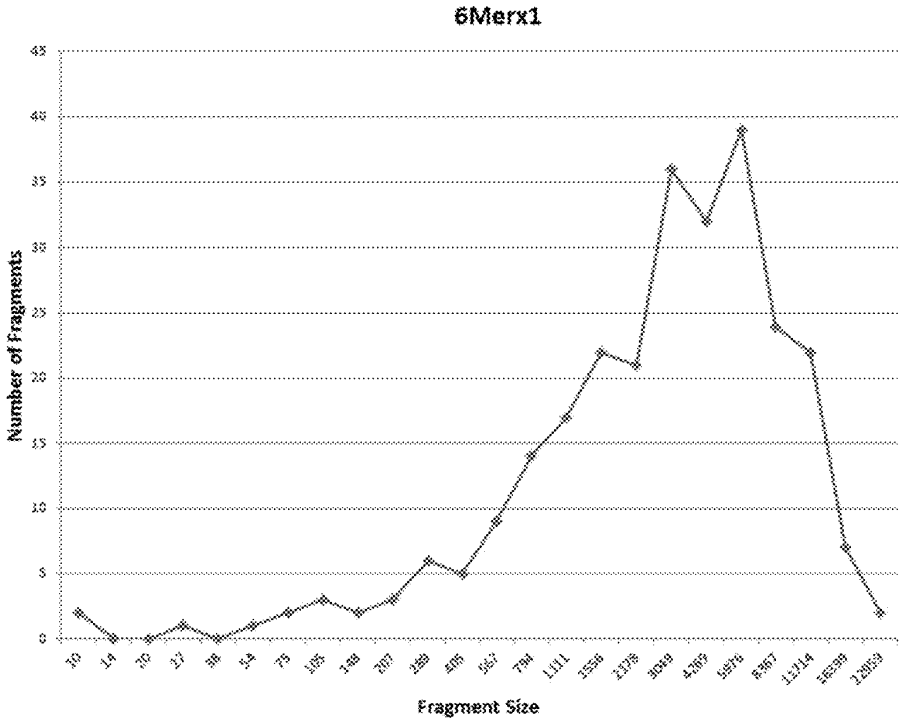


Fig. 3

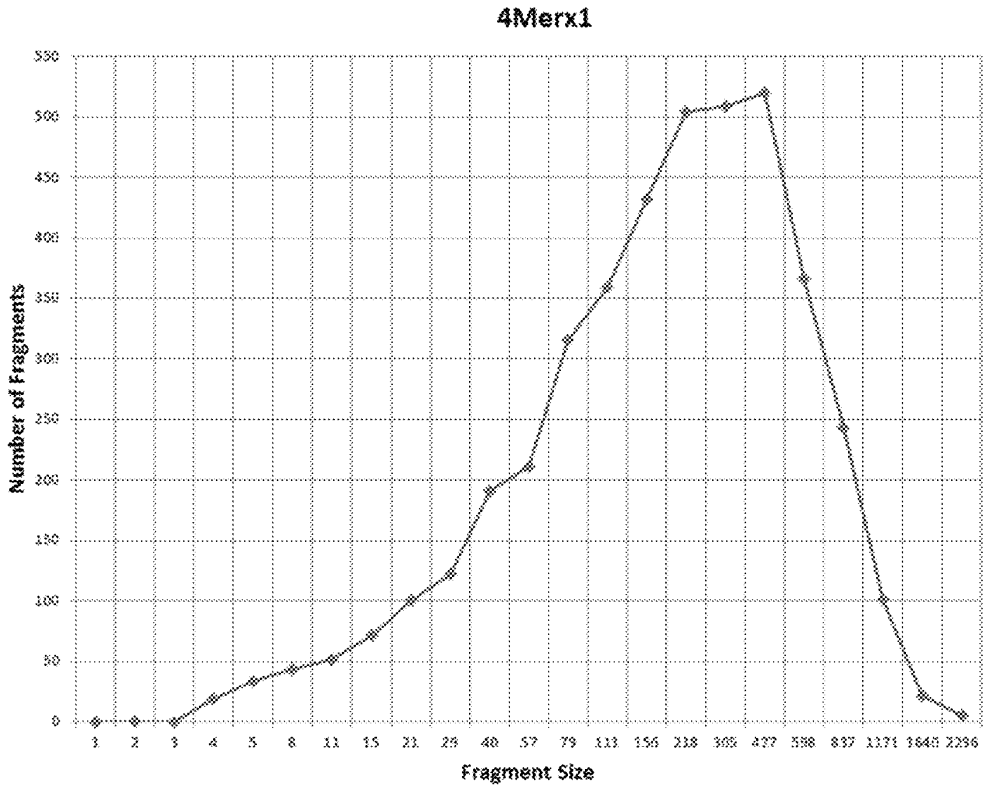
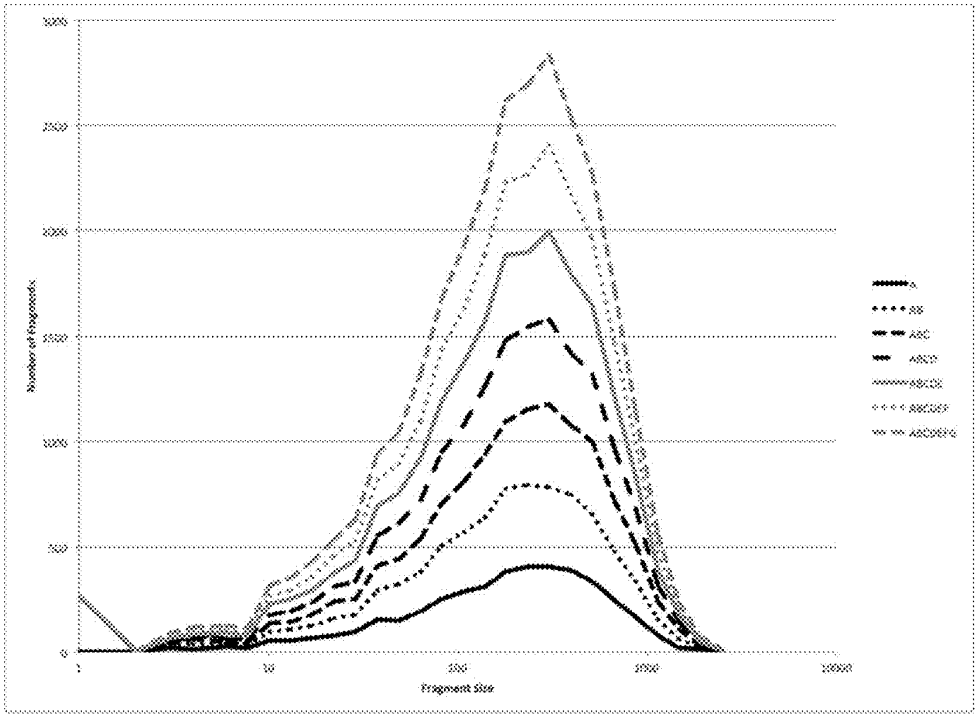

4Merx1
Number of Fragments
Fragment Size
0
50
100
150
200
250
300
350
400
450
500
550
1
2
3
4
5
8
11
15
21
29
40
57
79
113
156
218
302
477
598
837
1171
1640
2296

Fig. 4



A: CviQI	G/TAC
B: BfaI	C/TAG
C: HinP1I	G/CGC
D: CviAII	C/ATG
E: TaqαI	T/CGA
F: MseI	T/TAA
G: MspI	C/CGG

Fig. 5

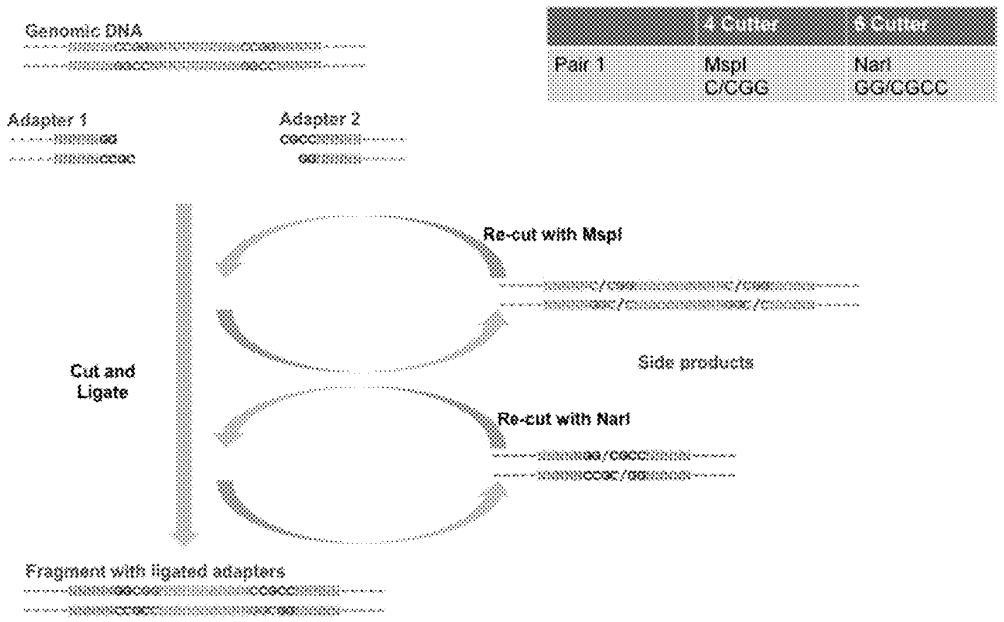

Genomic DNA
Adapter 1
Adapter 2
4 Cutter
6 Cutter
Pair 1
MspI
C/CGG
NarI
GG/CGCC
Re-cut with MspI
Cut and
Ligate
Side products
Re-cut with NarI
Fragment with ligated adapters

Fig. 6

	4 Cutter	6 Cutter
Pair 1	MspI C/CGG	NarI GG/CGCC
Pair 2	BfaI C/TAG	NdeI CA/TATG
Pair 3	HinP1I G/CGC	ClaI AT/CGAT
Pair 4	MseI T/TAA	NdeI CA/TATG
Pair 5	CviQI G/TAC	NdeI CA/TATG
Pair 6	TaqαI T/CGA	AclI AA/CGTT

Fig. 7A

	4 Cutter	6 Cutter
Pair 1	RsaI GT/AC	PmeI GTTT/AAAC
Pair 2	AluI AG/CT	EcoRV GAT/ATC
Pair 3	BstUI CG/CG	PmeI GTTT/AAAC
Pair 4	DpnI GA/TC	StuI AGG/CCT
Pair 5	HaeIII GG/CC	PmeI GTTT/AAAC
Pair 6	HpyCH4V TG/CA	SfoI GGC/GCC

Fig. 7B

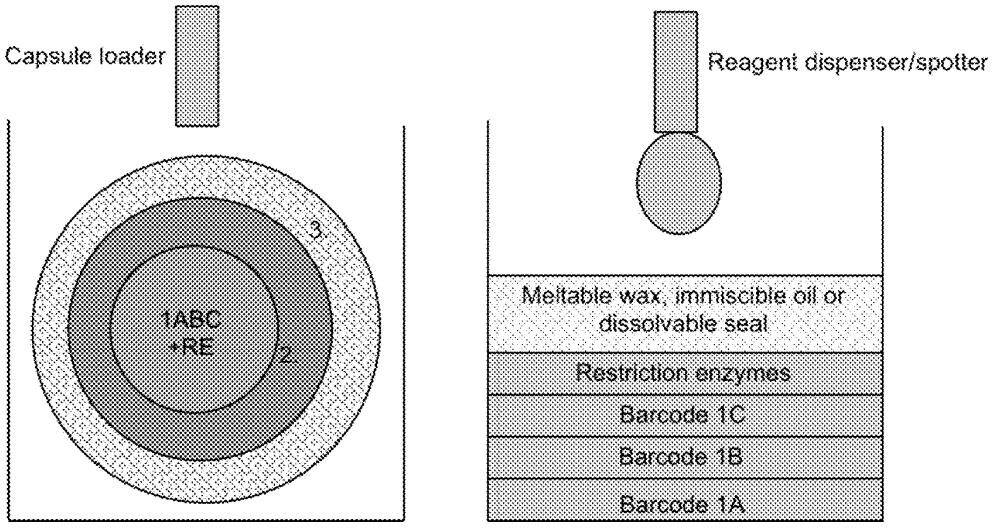

Capsule loader
3
1ABC
+RE
2
Reagent dispenser/spotter
Meltable wax, immiscible oil or dissolvable seal
Restriction enzymes
Barcode 1C
Barcode 1B
Barcode 1A

Fig. 8

METHODS AND SYSTEMS FOR PROCESSING POLYNUCLEOTIDES

CROSS-REFERENCE

This application is a continuation of U.S. patent application Ser. No. 15/376,582, filed Dec. 12, 2016, which is a continuation-in-part of U.S. patent application Ser. No. 14/104,650, filed on Dec. 12, 2013, now U.S. Pat. No. 9,567,631, which claims priority to U.S. Provisional Application No. 61/737,374, filed on Dec. 14, 2012; U.S. patent application Ser. No. 15/376,582 is also a continuation-in-part of U.S. patent application Ser. No. 14/250,701, filed on Apr. 11, 2014, which is a continuation of U.S. patent application Ser. No. 14/175,973, filed on Feb. 7, 2014, now U.S. Pat. No. 9,388,465, which claims priority to U.S. Provisional Application No. 61/844,804, filed on Jul. 10, 2013, U.S. Provisional Application No. 61/840,403, filed on Jun. 27, 2013, U.S. Provisional Application No. 61/800,223, filed on Mar. 15, 2013, and U.S. Provisional Application No. 61/762,435, filed on Feb. 8, 2013, each of which is entirely incorporated herein by reference for all purposes.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Jan. 6, 2017, is named 43487703501SL.txt and is 5 kilobytes in size.

BACKGROUND

The processing of polynucleotides and polynucleotide fragments is a critical aspect of a wide variety of technologies, including polynucleotide sequencing. Polynucleotide sequencing continues to find more widespread use in medical applications such as genetic screening and genotyping of tumors. Many polynucleotide sequencing methods rely on sample processing techniques solely utilizing random fragmentation of polynucleotides. Such random, uncontrolled fragmentation can introduce several problems in downstream processing. For example, these methods may produce fragments with large variation in length, including a large number or fraction of sequences that are too long to be sequenced accurately. This results in a loss of sequence information. Current methods of processing may also damage polynucleotides, resulting in incorrect sequence information, and/or the loss of sequence information. These, and other, problems may be significantly amplified by relatively minor operator variability. Thus, there is a significant need for improved methods that provide better control over all aspects of polynucleotide fragmentation and processing. In particular, there is need for polynucleotide processing methods that consistently provide fragments of appropriate size and composition for any downstream application, including sequencing.

SUMMARY

I. Non-Overlapping Fragmentation

This disclosure provides methods, compositions, systems, and devices for processing polynucleotides. In one example, a method provided herein comprises: (a) providing a target polynucleotide; (b) fragmenting said target polynucleotide to generate a plurality of non-overlapping first polynucleotide fragments; (c) partitioning said first polynucleotide fragments to generate partitioned first polynucleotide fragments, wherein at least one partition of said partitioned first polynucleotide fragments comprises a first polynucleotide fragment with a unique sequence within said at least one partition; and (d) fragmenting said partitioned first polynucleotide fragments, to generate a plurality of non-overlapping second polynucleotide fragments.

In some of the methods provided in this disclosure, a third and fourth set of polynucleotide fragments are generated by performing the method described above and additionally performing a method comprising: (a) fragmenting said target polynucleotide to generate a plurality of non-overlapping third polynucleotide fragments; (b) partitioning said third polynucleotide fragments to generate partitioned third polynucleotide fragments, wherein at least one partition of said partitioned third polynucleotide fragments comprises a third polynucleotide fragment with a unique sequence within said at least one partition; and (c) fragmenting said partitioned third polynucleotide fragments to generate a plurality of non-overlapping fourth polynucleotide fragments.

The third polynucleotide fragments may overlap with the first polynucleotide fragments. The fourth polynucleotide fragments may overlap with the second polynucleotide fragments.

The target polynucleotide may be, for example, DNA, RNA, cDNA, or any other polynucleotide.

In some cases, at least one of the first, second, third, and fourth polynucleotide fragments are generated by an enzyme. The enzyme may be a restriction enzyme. The restriction enzyme used to generate the first polynucleotide fragments may be different from the restriction enzyme used to generate the third polynucleotide fragments. The restriction enzyme used to generate the second polynucleotide fragments may be different from the restriction enzyme used to generate the fourth polynucleotide fragments. The restriction enzymes may have a recognition site of at least about six nucleotides in length.

The fragments can be of a variety of lengths. For example, the first and/or third polynucleotide fragments may have a median length of least about 10,000 nucleotides. The second or fourth polynucleotide fragments may have a median length of less than about 200 nucleotides.

The fragments can be attached to barcodes. For example, the second polynucleotide fragments and/or the fourth polynucleotide fragments may be attached to barcodes, to generate barcoded second and/or fourth polynucleotide fragments. The barcodes may be polynucleotide barcodes. The attachment of the barcodes to the polynucleotide fragments may be performed using an enzyme. The enzyme may be a ligase. The barcoded fragments may be pooled. Unpooled or pooled barcoded fragments may be sequenced.

In some cases, one or more steps of the methods described in this disclosure may be performed within a device. The device may comprise at least one well. The well may be a microwell. Any of the partitioning steps described in this disclosure may be performed by dispensing into a microwell.

The microwell (or well) may comprise reagents. These reagents may be any reagent, including, for example, barcodes, enzymes, adapters, and combinations thereof. The reagents may be physically separated from a polynucleotide sample placed in the microwell. This physical separation may be accomplished by containing the reagents within a microcapsule that is placed within a microwell. The physical separation may also be accomplished by dispensing the reagents in the microwell and overlaying the reagents with a layer that is, for example, dissolvable, meltable, or per-

meable prior to introducing the polynucleotide sample into the microwell. This layer may be, for example, an oil, wax, membrane, or the like. The microwell may be sealed at any point, for example after addition of the microcapsule, after addition of the reagents, or after addition of either of these components plus a polynucleotide sample.

Partitioning may also be performed by a variety of other means, including through the use of fluid flow in microfluidic channels, by emulsification, using spotted arrays, by surface acoustic waves, and by piezoelectric droplet generation.

Additional methods of fragmenting nucleic acids that are compatible with the methods provided herein include mechanical disruption, sonication, chemical fragmentation, treatment with UV light, and heating, and combinations thereof. These methods may be used to fragment, for example, the partitioned first or third polynucleotide fragments described above.

Partitioning may be done at any time. For example, the first polynucleotide fragments and/or the third polynucleotide fragments may each be further partitioned into two or more partitions before further processing.

Pseudo-Random Fragmentation

This disclosure provides methods for pseudo-random fragmentation of polynucleotides. In some cases, such methods comprise: (a) providing a target polynucleotide; (b) fragmenting said target polynucleotide to generate a plurality of first polynucleotide fragments; (c) partitioning said first polynucleotide fragments to generate partitioned first polynucleotide fragments, such that at least one partition comprises a first polynucleotide fragment with a unique sequence within said at least one partition; and (d) fragmenting said partitioned first polynucleotide fragments with at least one restriction enzyme in at least one partition, to generate a plurality of second polynucleotide fragments, wherein said partitioned first polynucleotide fragment is fragmented with at least two restriction enzymes across all partitions.

In some cases, at least two restriction enzymes are disposed within the same partition. In some cases, at least two restriction enzymes are disposed across a plurality of different partitions.

The pseudo-random fragmentation methods can be performed in order to yield fragments of a certain size. In some cases, at least about 50% of the nucleotides within a target polynucleotide are within about 100 nucleotides of a restriction site of a restriction enzyme used to perform pseudo-random fragmentation. In some cases, at most about 25% of the nucleotides within a target polynucleotide are within about 50 nucleotides of a restriction site of a restriction enzyme used to perform pseudo-random fragmentation. In some cases, at most about 10% of the nucleotides within a target polynucleotide are more than about 200 nucleotides from a restriction site a restriction enzyme used to perform pseudo-random fragmentation.

A polynucleotide may be treated with two or more restriction enzymes concurrently or sequentially.

The pseudo-randomly fragmented polynucleotides may be attached to barcodes, to generate barcoded polynucleotide fragments. The barcoded polynucleotides may be pooled and sequenced.

The number of partitions holding the partitioned first polynucleotide fragments may be at least about 1,000 partitions. The volume of these partitions may be less than about 500 nanoliters.

Each enzyme may occupy an equivalent number of partitions, or each enzyme may occupy a different number of partitions.

III. Restriction Enzyme-Mediated Recycling

This disclosure provides methods for recycling certain unwanted reaction side products back into starting materials that can be used to generate a desired product. In some cases, these methods comprise: (a) providing a first polynucleotide, a second polynucleotide, a first restriction enzyme, and a second restriction enzyme, wherein said first polynucleotide comprises a target polynucleotide or a fragment thereof; and (b) attaching said first polynucleotide to said second polynucleotide, to generate a polynucleotide product, wherein said first restriction enzyme cuts a polynucleotide generated by attachment of said first polynucleotide to itself, said second restriction enzyme cuts a polynucleotide generated by attachment of said second polynucleotide to itself, and neither said first restriction enzyme nor said second restriction enzyme cuts said polynucleotide product.

The first polynucleotide may be generated in the same reaction volume as the polynucleotide product, or in a different reaction volume. The target polynucleotide may be, for example, a fragment of genomic DNA.

The second polynucleotide may be generated in the same reaction volume as the polynucleotide product, or in a different reaction volume. The second polynucleotide may be, for example, a barcode or an adapter.

The first restriction enzyme may have a recognition site of at most about four nucleotides in length. The second restriction enzyme may have a recognition site of at least about six nucleotides in length. The first restriction enzyme may have a recognition site of about four nucleotides in length. The second restriction enzyme may have a recognition site of at least about five nucleotides in length.

The first and second restriction enzymes may generate ligation compatible ends. These ends may have single-stranded overhangs (i.e., "sticky ends") or be blunt. The sticky ends may match in sequence and orientation, to allow ligation. The attachment step may be performed by ligation.

The sequence 5' to the ligation compatible end generated by the first restriction enzyme may be different from the sequence 5' to the ligation compatible end generated by the second restriction enzyme. This will ensure that the desired product cannot be re-cut by either restriction enzyme.

The sequence 3' to the ligation compatible end generated by the first restriction enzyme may be different from the sequence 3' to the ligation compatible end generated by the second restriction enzyme. This will ensure that the desired product cannot be re-cut by either restriction enzyme. Given the criteria provided throughout this specification, one of ordinary skill in the art will recognize that many pairs of enzymes are suitable for use with this method.

The recycling may provide increased yield of the desired product, for example at least about 75% (w/w).

Also provided by this disclosure is a polynucleotide fragment generated by any of the methods provided herein, devices for performing the methods provided herein, and systems for performing the methods provided herein.

The methods provided in this disclosure (and portions thereof) may also be used with each other. For example, the non-overlapping fragmentation methods may be used alone and/or with the pseudo-random fragmentation methods and/or with the restriction enzyme-mediated recycling methods. Likewise, the pseudo-random fragmentation methods may be used alone and/or with the non-overlapping fragmentation methods and/or with the restriction enzyme-mediated recycling methods. Similarly, the restriction enzyme-medi-

ated recycling methods may be used alone and/or with the non-overlapping fragmentation methods and/or with the pseudo-random fragmentation methods.

Additional aspects and advantages of the present disclosure will become readily apparent to those skilled in this art from the following detailed description, wherein only illustrative embodiments of the present disclosure are shown and described. As will be realized, the present disclosure is capable of other and different embodiments, and its several details are capable of modifications in various obvious respects, all without departing from the disclosure. Accordingly, the drawings and description are to be regarded as illustrative in nature, and not as restrictive.

INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

The novel features of methods, compositions, systems, and devices of this disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of this disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the methods, compositions, systems, and devices of this disclosure are utilized, and the accompanying drawings of which:

FIG. **1** is a schematic representation of overlapping and non-overlapping deoxyribonucleic acid (DNA) fragments.

FIG. **2** is a schematic representation of methods of generating non-overlapping DNA fragments for DNA sequencing. FIG. **2** discloses SEQ ID NOS 8-10, respectively, in order of appearance.

FIG. **3** shows a distribution of DNA fragment size after simulating generation of 1 Mbp random DNA sequences followed by cutting the sequences with a 6Mer cutter, StuI (AGG/CCT).

FIG. **4** shows a distribution of DNA fragment size after simulating generation of 1 Mbp random DNA sequences followed by cutting the sequences with a 4Mer cutter, CviQI (G/TAC).

FIG. **5** shows a distribution of DNA fragment size after simulating the generation of a 1 Mbp random DNA sequence followed by cutting the sequences with seven 4Mer cutters: (1) CviQI (G/TAC), (2) BfaI (C/TAG), (3) HinP1I (G/CGC), (4) CviAII (C/ATG), (5) TaqαI (T/CGA), (6) MseI (T/TAA), and (7) MspI (C/CGG).

FIG. **6** shows the generation of unwanted byproducts ("Side products") during ligation of adapters to genomic DNA fragments and the recycling of the unwanted byproducts into starting materials ("Genomic DNA", "Adapter 1", and "Adapter 2") by paring of appropriate restriction enzymes (here, MspI and NarI). FIG. **6** discloses SEQ ID NOS 11 and 11-13, respectively, in order of appearance.

FIG. **7**A shows exemplary 4Mer cutter and 6Mer cutter pairs generating sticky ends.

FIG. **7**B shows exemplary 4Mer cutter and 6Mer cutter pairs generating blunt ends.

FIG. **8** shows a capsule containing reagents for barcoding of polynucleotide fragments in a microwell (left) and a microwell containing reagents for barcoding of polynucleotide fragments dispensed in a microwell and sealed to prevent evaporation (right).

DETAILED DESCRIPTION

While various embodiments of the invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions may occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed.

This disclosure provides methods, compositions, systems, and devices for processing polynucleotides. Applications include processing polynucleotides for polynucleotide sequencing. Polynucleotides sequencing includes the sequencing of whole genomes, detection of specific sequences such as single nucleotide polymorphisms (SNPs) and other mutations, detection of nucleic acid (e.g., deoxyribonucleic acid) insertions, and detection of nucleic acid deletions.

Utilization of the methods, compositions, systems, and devices described herein may incorporate, unless otherwise indicated, conventional techniques of organic chemistry, polymer technology, microfluidics, molecular biology and recombinant techniques, cell biology, biochemistry, and immunology. Such conventional techniques include microwell construction, microfluidic device construction, polymer chemistry, restriction digestion, ligation, cloning, polynucleotide sequencing, and polynucleotide sequence assembly. Specific, non-limiting, illustrations of suitable techniques are described throughout this disclosure. However, equivalent procedures may also be utilized. Descriptions of certain techniques may be found in standard laboratory manuals, such as *Genome Analysis: A Laboratory Manual Series* (*Vols. I-IV*), *Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), and "Oligonucleotide Synthesis: A Practical Approach" 1984, IRL Press London, all of which are herein incorporated in their entirety by reference for all purposes.

I. Definitions

The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including", "includes", "having", "has", "with", "such as", or variants thereof, are used in either the specification and/or the claims, such terms are not limiting and are intended to be inclusive in a manner similar to the term "comprising".

The term "about," as used herein, generally refers to a range that is 15% greater than or less than a stated numerical value within the context of the particular usage. For example, "about 10" would include a range from 8.5 to 11.5.

The term "barcode", as used herein, generally refers to a label that may be attached to a polynucleotide, or any variant thereof, to convey information about the polynucleotide. For example, a barcode may be a polynucleotide sequence attached to all fragments of a target polynucleotide contained within a particular partition. This barcode may then be sequenced with the fragments of the target polynucle-

otide. The presence of the same barcode on multiple sequences may provide information about the origin of the sequence. For example, a barcode may indicate that the sequence came from a particular partition and/or a proximal region of a genome. This may be particularly useful when several partitions are pooled before sequencing.

The term "bp," as used herein, generally refers to an abbreviation for "base pairs".

The term "Mer," as used herein to refer to restriction enzymes, generally refers to the number of nucleotides in one strand of a restriction enzyme's recognition site. For example, the enzyme CviQI has a recognition site of GTAC (4 nucleotides on one strand) and is thus referred to as a "4Mer cutter." The enzyme StuI has a recognition site of AGGCCT (6 nucleotides on one strand) and is thus referred to as a "6Mer cutter."

The term "microwell," as used herein, generally refers to a well with a volume of less than 1 mL. Microwells may be made in various volumes, depending on the application. For example, microwells may be made in a size appropriate to accommodate any of the partition volumes described herein.

The terms "non-overlapping" and "overlapping," as used to refer to polynucleotide fragments, generally refer to a collection of polynucleotide fragments without overlapping sequence or with overlapping sequence, respectively. By way of illustration, consider a hypothetical partition containing three copies of a genome (FIG. **1**, top set of sequences). This genome may be fragmented randomly (e.g., by shearing in a pipette) or non-randomly (e.g., by digesting with a rare cutter). Fragmenting randomly produces overlapping sequences (second set of sequences from top in FIG. **1**, "Fragmented randomly to generate overlap"), because each copy of the genome is cut at different positions. After sequencing of the fragments (which provides "sequence contigs"), this overlap may be used to determine the linear order of the fragments, thereby enabling assembly of the entire genomic sequence. By contrast, fragmenting by digesting with a rare cutter produces non-overlapping fragments, because each copy of the (same) genome is cut at the same position (third set of sequences from the top in FIG. **1**, "Fragmented non-randomly using RE-1 to generate non-overlapping fragments"). After sequencing these fragments, it may be difficult to deduce their linear order due to the lack of overlap between the fragments. However, as described in this disclosure, the linear order may be determined by, for example, fragmenting the genome using a different technique. The fourth set of sequences from the top of FIG. **1** demonstrates the use of a second rare-cutter enzyme to generate a second set of non-overlapping fragments ("Fragmented non-randomly using RE-2 to generate non-overlapping fragments"). Because two different enzymes, for example, are used to generate the two sets of non-overlapping fragments, there is overlap between the fragments generated with the first rare-cutter enzyme (RE-1) and the fragments generated with the second rare-cutter enzyme (RE-2). This overlap may then be used to assemble the linear order of the sequences, and therefore the sequence of the entire genome.

The term "partition," as used herein, may be a verb or a noun. When used as a verb (e.g., "partitioning"), the term refers to the fractionation of a substance (e.g., a polynucleotide) between vessels that can be used to sequester one fraction from another. Such vessels are referred to using the noun "partition." Partitioning may be performed, for example, using microfluidics, dilution, dispensing, and the like. A partition may be, for example, a well, a microwell, a droplet, a test tube, a spot, or any other means of sequestering one fraction of a sample from another. In the methods and systems described herein, polynucleotides are often partitioned into microwells.

The terms "polynucleotide" or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides. Exemplary polynucleotides include deoxyribonucleic acids, ribonucleic acids, and synthetic analogues thereof, including peptide nucleic acids.

The term "rare-cutter enzyme," as used herein, generally refers to an enzyme with a recognition site that occurs only rarely in a genome. The size of restriction fragments generated by cutting a hypothetical random genome with a restriction enzyme may be approximated by 4^N, where N is the number of nucleotides in the recognition site of the enzyme. For example, an enzyme with a recognition site consisting of 7 nucleotides would cut a genome once every 4^7 bp, producing fragments of about 16,384 bp. Generally rare-cutter enzymes have recognition sites comprising 6 or more nucleotides. For example, a rare cutter enzyme may have a recognition site comprising or consisting of 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 nucleotides. Examples of rare-cutter enzymes include NotI (GCGGCCGC), XmaIII (CGGCCG), SstII (CCGCGG), SalI (GTCGAC), NruI (TCGCGA), NheI (GCTAGC), Nb.BbvCI (CCTCAGC), BbvCI (CCTCAGC), AscI (GGCGCGCC), AsiSI (GCGATCGC), FseI (GGCCGGCC), PacI (TTAATTAA), PmeI (GTTTAAAC), SbfI (CCTGCAGG), SgrAI (CRCCGGYG), SwaI (ATTTAAAT), BspQI (GCTCTTC), SapO (GCTCTTC), SfiI (GGCCNNNNNGGCC (SEQ ID NO: 1)), CspCI (CAANNNNNGTGG (SEQ ID NO: 2)), AbsI (CCTCGAGG), CciNI (GCGGCCGC), FspAI (RTGCGCAY), MauBI (CGCGCGCG), MreI (CGCCGGCG), MssI (GTTTAAAC), PalAI (GGCGCGCC), RgaI (GCGATCGC), RigI (GGCCGGCC), SdaI (CCTGCAGG), SfaAI (GCGATCGC), SgfI (GCGATCGC), SgrDI (CGTCGACG), SgsI (GGCGCGCC), SmiI (ATTTAAAT), SrfI (GCCCGGGC), Sse2321 (CGCCGGCG), Sse83871 (CCTGCAGG), LguI (GCTCTTC), PciSI (GCTCTTC), AarI (CACCTGC), AjuI (GAANNNNNNNTTGG (SEQ ID NO: 3)), AloI (GAACNNNNNNTCC (SEQ ID NO: 4)), BadI (GAAGNNNNNNTAC (SEQ ID NO: 4)), PpiI (GAACNNNNNCTC (SEQ ID NO: 6)), PsdI (GAACNNNNNNTAC (SEQ ID NO: 7)), and others.

The term "target polynucleotide," as used herein, generally refers to a polynucleotide to be processed. For example, if a user intends to process genomic DNA into fragments that may be sequenced, the genomic DNA would be the target polynucleotide. If a user intends to process fragments of a polynucleotide, then the fragments of the polynucleotide may be the target polynucleotide.

II. Non-Overlapping Fragmentation

This disclosure provides methods, compositions, systems, and devices for the generation of non-overlapping polynucleotide fragments. These fragments may be useful for downstream analyses such as DNA sequencing. For example, with reference to FIG. **2**, a target polynucleotide **101**, such as genomic DNA, may be fragmented to generate a plurality of non-overlapping first polynucleotide fragments **102**. This fragmentation may be performed, for example, by digesting the target polynucleotide with a rare-cutter enzyme (e.g., rare-cutter enzyme 1), or an artificial restriction DNA cutter (ARCUT; Yamamoto et al., *Nucleic Acids Res.*, 2007, 35(7), e53). The first polynucleotide fragments may then be partitioned, such that at least one partition **103** comprises a first polynucleotide fragment with a unique sequence within that partition and, optionally, an additional first polynucle-

otide fragment with a different sequence **104**. The partitioned first polynucleotide fragments may then be further fragmented to produce a plurality of non-overlapping second polynucleotide fragments **105**. This fragmentation may be performed, for example, by enzymatic digestion, exposure to ultraviolet (UV) light, ultrasonication, and/or mechanical agitation. The second polynucleotide fragments may be of a size that is appropriate for DNA sequencing, i.e., a size that enables a DNA sequencer to obtain accurate sequence data for the entire fragment.

In order to facilitate DNA sequence assembly, the second fragments may be attached to a barcode, which may be attached to all of the second fragments disposed in a particular partition. The barcode may be, for example, a DNA barcode. With continued reference to FIG. **2**, after attachment of the barcode, the barcoded fragments may be pooled into a partition comprising pooled, barcoded sequences **106**. Three barcodes are depicted as [1], [2], and [3] in **106**. The pooled fragments may be sequenced.

Certain methods of genome sequence assembly rely on the presence of overlapping fragments in order to generate higher order sequence data (e.g., whole genome sequences) from sequenced fragments. The methods, compositions, systems, and devices provided herein may also be used to provide overlapping fragments. For example, with continued reference to FIG. **2**, fragments overlapping with the first and second fragments described above may be generated by generating a plurality of non-overlapping third polynucleotide fragments from the target polynucleotide **107**. The third polynucleotide fragments may be generated, for example, by digesting the target polynucleotide **101** with a rare-cutter enzyme (e.g., rare-cutter enzyme 2; or ARCUT) that is different from the rare-cutter enzyme used to generate the first polynucleotide fragments. If rare-cutter enzymes 1 and 2 are chosen to cut the target polynucleotide sequence at different positions, the third polynucleotide fragments and the first polynucleotide fragments will overlap. The third polynucleotide fragments may then be processed as described above for the first polynucleotide fragments.

Specifically, the third polynucleotide fragments may be partitioned such that at least one partition **108** comprises a third polynucleotide fragment with a unique sequence within that partition and, optionally, an additional third polynucleotide fragment with a different sequence **109**. These partitioned fragments may then be further fragmented to produce a plurality of non-overlapping fourth polynucleotide fragments **110**. The fourth polynucleotides fragments and the second polynucleotide fragments may overlap. As for the second polynucleotide fragments, the fourth polynucleotide fragments may be generated by, for example, enzymatic digestion, exposure to ultraviolet (UV) light, ultrasonication, and/or mechanical agitation. The fourth fragments may be of a size that is appropriate for DNA sequencing, i.e., a size that enables a DNA sequencer to obtain accurate sequence data for the entire fragment.

In order to facilitate DNA sequencing, the fourth fragments may be attached to a barcode, which may be attached to all of the fourth fragments disposed in a particular partition. The barcode may be, for example, a DNA barcode. After attachment of the barcode, the barcoded fragments may be pooled, into a partition comprising pooled, barcoded, sequences **111**. Three barcodes are depicted as [4], [5], and [6] in **111**. The pooled fragments may be sequenced. The overlap between the sequences of the second fragments and the fourth fragments may be used to assemble higher order sequences, such whole genome sequences.

The steps described above may be performed using a variety of techniques. For example, certain steps of the methods may be performed in a device comprising microwell chambers (microwells), for example a microfluidic device. These microwells may be connected to each other, or to a source of reagents, by channels. The first and third fragments may be generated outside of the device and then introduced into the device (or separate devices) for further processing. Partitioning of the first and third fragments may accomplished using fluidic techniques. Generation of the second and fourth fragments may then occur within the microwells of the device or devices. These microwells may contain reagents for barcoding of the second and fourth fragments, such as DNA barcodes, ligase, adapter sequences, and the like. Microwells may feed or be directed into a common outlet, so that barcoded fragments may be pooled or otherwise collected into one or more aliquots which may then be sequenced.

In another example, the entire process could be performed within a single device. For example, a device could be split into two sections. A first section may comprise a partition comprising rare-cutter enzyme 1 (generating first polynucleotide fragments) and a second section may comprise a partition comprising rare-cutter enzyme 2 (generating third polynucleotide fragments). An aliquot of the target polynucleotide sequence may be placed into each of these partitions. Following digestion, the enzyme may be inactivated and the samples may be partitioned, fragmented, barcoded, pooled, and sequenced as described above. For convenience, this example has been described using rare-cutter enzymes as the means of generating the first and third fragments. However, this is not intended to be limiting, here or anywhere else in this disclosure. One of ordinary skill in the art will readily recognize that other means of generating non-overlapping, or predominantly non-overlapping, fragments would be just as suitable as the use of rare-cutter enzymes.

III. Pseudo-Random Fragmentation

This disclosure also provides methods, compositions, systems, and devices for fragmenting polynucleotides in a pseudo-random manner. This may be performed by treating partitioned polynucleotides with more than one restriction enzyme. For example, polynucleotides partitioned into microwells may be treated with combinations of restriction enzymes. Within each partition containing a particular combination of enzymes, the cutting is defined and predictable. However, across all of the partitions (through the use of multiple combinations of restriction enzymes in different partitions), the polynucleotide fragments generated approximate those obtained from methods of random fragmentation. However, these polynucleotide fragments are generated in a much more controlled manner than random fragments generated by methods known in the art (e.g., shearing). The partitioned, pseudo-randomly fragmented polynucleotides may be barcoded, as described throughout this disclosure, pooled, and sequenced. The pseudo-random fragmentation methods may be used with the non-overlapping fragmentation methods described herein, or with any other method described herein such as the high yield adapter/barcode attachment method. Pseudo-random fragmentation may occur by exposing a polynucleotide to multiple enzymes simultaneously, sequentially, or simultaneously and sequentially.

Thus, this disclosure provides methods and systems for processing polynucleotides comprising generating pseudo-random fragments of said polynucleotides. These pseudo random fragments are generated by treating a polynucleotide

with more than one restriction enzyme. For example, a polynucleotide may be treated with about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, 50, or more restriction enzymes. A polynucleotide may be treated with at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, 50, or more restriction enzymes. A polynucleotide may be treated with at least 2 but fewer than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, or 50 restriction enzymes. A polynucleotide may be treated with about 2-4, 4-6, 6-8, 8-10, 10-12, 12-14, 14-16, 16-18, 18-20, 20-25, 25-30, 35-40, 40-45, or 45-50 restriction enzymes.

The restriction enzymes may be chosen in order to maximize the number or fraction of fragments that will provide accurate sequencing data, based on the size of the fragments generated by the pseudo-random fragmentation. For present day sequencing technology, accuracy degrades beyond a read length of about 100 nucleotides. Therefore, fragments of about 200 or fewer nucleotides generally provide the most accurate sequence data since they can be sequenced from either end. Fragments below about 50 nucleotides are generally less desirable because, although the produce accurate sequencing data, they underutilize the read length capacity of current sequencing instruments which are capable of 150 to 200 base reads. Fragments of about 200 to about 400 nucleotides may be sequenced with systematic errors introduced as the read length increases beyond the initial 100 bases from each end. Sequence information from fragments greater than about 400 nucleotides is typically completely lost for those bases greater than 200 bases from either end. One of skill in the art will recognize that sequencing technology is constantly advancing and that the ability to obtain accurate sequence information from longer fragments is also constantly improving. Thus, the pseudo-random fragmentation methods presented herein may be used to produce optimal fragment lengths for any sequencing method.

In some cases, fragments may be defined by the distance of their component nucleotides from a restriction site (measured in nucleotides). For example, each nucleotide within a polynucleotide fragment generated by the pseudo-random fragmentation method may be less than about 10, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 550, 600, 1000, 5000, 10000, or 100000 nucleotides from the restriction site of an enzyme to which the polynucleotide is exposed. Each nucleotide within a polynucleotide fragment may be about 10, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 550, 600, 1000, 5000, 10000, or 100000 nucleotides from the restriction site of an enzyme to which the polynucleotide is exposed. Each nucleotide within a polynucleotide fragment may be at least about 10, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 550, 600, 1000, 5000, 10000, or 100000 nucleotides from the restriction site of an enzyme to which the polynucleotide is exposed.

In some cases, at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, of the nucleotides comprising a target polynucleotide sequence are within about 10, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 550, 600, 1000, 5000, 10000, or 100000 nucleotides from the restriction site of an enzyme to which the polynucleotide is exposed. All combinations of these percentages and polynucleotide lengths are contemplated.

In some cases, at less than about 1%, 5%, 10%, 25%, 30%, 35%, 40%, 45%, or 50% of the nucleotides comprising a target polynucleotide sequence are within about 1, 5, 10, 50, 200, 250, 300, 350, 400, 550, 600, 1000, 5000, 10000, or 100000 nucleotides from the restriction site of an enzyme to which the polynucleotide is exposed. All combinations of these percentages and polynucleotide lengths are contemplated.

The pseudo-random fragmentation methods may be used to obtain fragments of about 10 to 50 nucleotides, 46 to 210 nucleotides, 50 to 250 nucleotides, 250 to 400 nucleotides, 400 to 550 nucleotides, 550 to 700 nucleotides, 700 to 1000 nucleotides, 1000 to 1300 nucleotides, 1300 to 1600 nucleotides, 1600 to 1900 nucleotides, 1900 to 2200 nucleotides, or 2200 to 3000 nucleotides. The pseudo-random fragmentation methods may be used to obtain fragments with a mean or median of about 40 nucleotides, 60 nucleotides, 80 nucleotides, 100 nucleotides, 120 nucleotides, 130 nucleotides, 140 nucleotides, 160 nucleotides, 180 nucleotides, 200 nucleotides, 250 nucleotides, 300 nucleotides, 400 nucleotides, 500 nucleotides, 600 nucleotides, 700 nucleotides, 800 nucleotides, 900 nucleotides, 1000 nucleotides, 1200 nucleotides, 1400 nucleotides, 1600 nucleotides, 1800 nucleotides, 2000 nucleotides, 2500 nucleotides, 3000 nucleotides, or more. The pseudo-random fragmentation methods may be used to obtain fragments with a mean or median of at least about 40 nucleotides, 60 nucleotides, 80 nucleotides, 100 nucleotides, 120 nucleotides, 130 nucleotides, 140 nucleotides, 160 nucleotides, 180 nucleotides, 200 nucleotides, 250 nucleotides, 300 nucleotides, 400 nucleotides, 500 nucleotides, 600 nucleotides, 700 nucleotides, 800 nucleotides, 900 nucleotides, 1000 nucleotides, 1200 nucleotides, 1400 nucleotides, 1600 nucleotides, 1800 nucleotides, 2000 nucleotides, 2500 nucleotides, 3000 nucleotides, or more. The pseudo-random fragmentation methods may be used to obtain fragments with a mean or median of less than about 40 nucleotides, 60 nucleotides, 80 nucleotides, 100 nucleotides, 120 nucleotides, 130 nucleotides, 140 nucleotides, 160 nucleotides, 180 nucleotides, 200 nucleotides, 250 nucleotides, 300 nucleotides, 400 nucleotides, 500 nucleotides, 600 nucleotides, 700 nucleotides, 800 nucleotides, 900 nucleotides, 1000 nucleotides, 1200 nucleotides, 1400 nucleotides, 1600 nucleotides, 1800 nucleotides, 2000 nucleotides, 2500 nucleotides, or 3000 nucleotides.

In some examples, the pseudo-random fragmentation methods provided herein are used to generate fragments wherein a particular percentage (or fraction) of the fragments generated fall within any of the size ranges described herein. For example, about 0%, 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18%, 20%, 22%, 24%, 26%, 28%, 30%, 32%, 34%, 36%, 38%, 40%, 42%, 44%, 46%, 48%, 50%, 52%, 54%, 56%, 58%, 60%, 62%, 64%, 66%, 68%, 70%, 72%, 74%, 76%, 78%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, 98%, or 100% of the fragments generated may fall within any of the size ranges described herein.

In some examples multiple 4Mer cutters may be used to provide a distribution of about 18% of fragments of about 50 nucleotides or less, about 38% of fragments of about 200 nucleotides or less, about 25% of fragments between about 200 and about 400 nucleotides, and about 37% of fragments greater than about 400 nucleotides (e.g., see FIG. **4**).

Additionally, the pseudo-random fragmentation method may be designed to minimize the percentage of fragments greater than a certain number of nucleotides in length, in order to minimize the loss of sequence information. For example, the method may be designed to yield less than about 0.1%, 0.5%, 1%, 2%, 5%, 10%, 20%, or 50% fragments greater than 100 nucleotides. The method may be designed to yield less than about 0.1%, 0.5%, 1%, 2%, 5%, 10%, 20%, or 50% fragments greater than 150 nucleotides.

The method may be designed to yield less than about 0.1%, 0.5%, 1%, 2%, 5%, 10%, 20%, or 50% fragments greater than 200 nucleotides. The method may be designed to yield less than about 0.1%, 0.5%, 1%, 2%, 5%, 10%, 20%, or 50% fragments greater than 300 nucleotides, and so on. As the ability of sequencing technologies to accurately read long DNA fragments increases, the pseudo-random fragmentation methods of the invention may be used to generate sequences suitable for any chosen read length.

Enzymes for use with the pseudo-random fragmentation method described herein may be chosen, for example, based on the length of their recognition site and their compatibility with certain buffer conditions (to allow for combination with other enzymes). Enzymes may also be chosen so that their cutting activity is methylation insensitive, or sensitive to methylation. For example, restriction enzymes with shorter recognition sites generally cut polynucleotides more frequently. Thus, cutting a target polynucleotide with a 6Mer cutter will generally produce more large fragments than cutting the same polynucleotide with a 4Mer cutter (e.g., compare FIGS. **3** and **4**). Cutting a target polynucleotide with a plurality of enzymes (e.g. 2, 3, 4, 5, 6, 7, or more) may produce a greater number or fraction of fragments in the optimal size range for DNA sequencing than cutting with a single enzyme (see FIG. **5**). Any restriction enzyme may be used with this method. Many are named in this specification, but others are known in the art.

This disclosure also provides methods of selecting a plurality of enzymes for pseudo-random fragmentation of a polynucleotide sequence. For example, a target polynucleotide may be exposed separately to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 restriction enzymes. The size distribution of the target polynucleotide fragments is then determined, for example, by electrophoresis. The combination of enzymes providing the greatest number of fragments that are capable of being sequenced can then be chosen. The method can also be carried out in silico.

The enzymes may be disposed within the same partition, or within a plurality of partitions. For example, any of the plurality of enzyme number described herein may be disposed within a single partition, or across partitions. For example, a polynucleotide may be treated with about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, 50, or more restriction enzymes in the same partition, or across partitions. A polynucleotide may be treated with at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, 50, or more restriction enzymes in the same partition, or across partitions. A polynucleotide may be treated with at least 2 but fewer than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, or 50 restriction enzymes in the same partition, or across partitions. A polynucleotide may be treated with about 2-4, 4-6, 6-8, 8-10, 10-12, 12-14, 14-16, 16-18, 18-20, 20-25, 25-30, 35-40, 40-45, or 45-50 restriction enzymes in the same partition, or across partitions.

The distribution of the restriction enzymes among the partitions will vary depending on the restriction enzymes, the target polynucleotide, and the desired fragment size. In some cases, each restriction enzyme may be distributed across an equivalent number of partitions, so that the number of partitions occupied by each restriction enzyme is equivalent. For example, if 10 restriction enzymes are used in a device containing 1,000 partitions, each enzyme may be present in 100 partitions. In other cases, each restriction enzyme may be distributed across a non-equivalent number of partitions, so that the number of partitions occupied by each restriction enzyme is not equivalent. For example, if 10 restriction enzymes are used in a device containing 1,000 partitions, enzymes 1-8 may be present in 100 partitions each, enzyme 9 may be present in 50 partitions, and enzyme 10 may be present in 150 partitions. Placement of restriction enzymes in an unequal number of partitions may be beneficial, for example, when an enzyme generates a desired product at a low yield. Placing this low-yield enzyme in more partitions will therefore expose more of the target polynucleotide to the enzyme, increasing the amount of the desired product (e.g., fragment of a certain size or composition) that can be formed from the enzyme. Such an approach may be useful for accessing portions of a target polynucleotide (e.g., a genome) that are not cut by enzymes producing polynucleotide fragments at a higher yield. The restriction site and efficiency of an enzyme, composition of the target polynucleotide, and efficiency and side-products generated by the enzyme may all be among the factors considered when determining how many partitions should receive a particular enzyme.

In some cases, different numbers of restriction enzymes may be used in a single partition and across all partitions. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, or 50 restriction enzymes or more may be used in each partition, while 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, or 50 restriction enzymes or more may be used across all partitions. All combinations of these numbers are included within the invention. Non-limiting specific examples include the use of 1 restriction enzyme per partition and 2, 3, 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; 2 restriction enzymes per partition and 3, 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; 3 restriction enzymes per partition and 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; 4 restriction enzymes per partition and 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; 5 restriction enzymes per partition and 6, 7, 8, 9, or 10 restriction enzymes across all partitions; 6 restriction enzymes per partition and 7, 8, 9, or 10 restriction enzymes across all partitions; 7 restriction enzymes per partition and 8, 9, or 10 restriction enzymes across all partitions; 8 restriction enzymes per partition and 9 or 10 restriction enzymes across all partitions; and 9 restriction enzymes per partition and 10 or more restriction enzymes across all partitions.

In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, or 50 restriction enzymes or more may be used in each partition, while at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, or 50 restriction enzymes or more may be used across all partitions. All combinations of these numbers are included within the invention. Non-limiting specific examples include the use of at least 1 restriction enzyme per partition and at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at least 2 restriction enzymes per partition and at least 3, 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at least 3 restriction enzymes per partition and at least 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at least 4 restriction enzymes per partition and at least 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at least 5 restriction enzymes per partition and at least 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at least 6 restriction enzymes per partition and at least 7, 8, 9, or 10 restriction enzymes across all partitions; at least 7 restriction enzymes per partition and at least 8, 9, or 10 restriction enzymes across all partitions; at least 8 restriction enzymes per

partition and at least 9 or 10 restriction enzymes across all partitions; and at least 9 restriction enzymes per partition and at least 10 or more restriction enzymes across all partitions.

In some cases, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, or 50 restriction enzymes or more may be used in each partition, while at most 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, or 50 restriction enzymes or more may be used across all partitions. All combinations of these numbers are included within the invention. Non-limiting specific examples include the use of at most 1 restriction enzyme per partition and at most 2, 3, 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at most 2 restriction enzymes per partition and at most 3, 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at most 3 restriction enzymes per partition and at most 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at most 4 restriction enzymes per partition and at most 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at most 5 restriction enzymes per partition and at most 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at most 6 restriction enzymes per partition and at most 7, 8, 9, or 10 restriction enzymes across all partitions; at most 7 restriction enzymes per partition and at most 8, 9, or 10 restriction enzymes across all partitions; at most 8 restriction enzymes per partition and at most 9 or 10 restriction enzymes across all partitions; and at most 9 restriction enzymes per partition and at most 10 or more restriction enzymes across all partitions.

In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, or 50 restriction enzymes or more may be used in each partition, while at most 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, or 50 restriction enzymes or more may be used across all partitions. All combinations of these numbers are included within the invention. Non-limiting specific examples include the use of at least 1 restriction enzyme per partition and at most 2, 3, 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at least 2 restriction enzymes per partition and at most 3, 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at least 3 restriction enzymes per partition and at most 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at least 4 restriction enzymes per partition and at most 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at least 5 restriction enzymes per partition and at most 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at least 6 restriction enzymes per partition and at most 7, 8, 9, or 10 restriction enzymes across all partitions; at least 7 restriction enzymes per partition and at most 8, 9, or 10 restriction enzymes across all partitions; at least 8 restriction enzymes per partition and at most 9 or 10 restriction enzymes across all partitions; and at least 9 restriction enzymes per partition and at most 10 or more restriction enzymes across all partitions.

In some cases, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, or 50 restriction enzymes or more may be used in each partition, while at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 45, or 50 restriction enzymes or more may be used across all partitions. All combinations of these numbers are included within the invention. Non-limiting specific examples include the use of at most 1 restriction enzyme per partition and at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at most 2 restriction enzymes per partition and at least 3, 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at most 3 restriction enzymes per partition and at least 4, 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at most 4 restriction enzymes per partition and at least 5, 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at most 5 restriction enzymes per partition and at least 6, 7, 8, 9, or 10 restriction enzymes across all partitions; at most 6 restriction enzymes per partition and at least 7, 8, 9, or 10 restriction enzymes across all partitions; at most 7 restriction enzymes per partition and at least 8, 9, or 10 restriction enzymes across all partitions; at most 8 restriction enzymes per partition and at least 9 or 10 restriction enzymes across all partitions; and at most 9 restriction enzymes per partition and at least 10 or more restriction enzymes across all partitions.

IV. Restriction Enzyme-Mediated Recycling

As described throughout this disclosure, certain methods of the invention involve the addition of barcodes, adapters, or other sequences to fragmented target polynucleotides. Barcodes may be polynucleotide barcodes, which may be ligated to the fragmented target polynucleotides or added via an amplification reaction. As described throughout this disclosure, fragmentation of target polynucleotides may be performed using one or more restriction enzymes contained within a partition (e.g., a microwell) where the fragmentation is performed. The partition may also contain a polynucleotide barcode and a ligase, which enables the attachment of the barcode to the fragmented polynucleotide. In some cases, an adapter may be used to make a fragmented target polynucleotide compatible for ligation with a barcode. The presence of adapters, fragmented target polynucleotide, barcodes, restriction enzymes, and ligases in the same partition may lead to the generation of undesirable side products that decrease the yield of a desired end product. For example, self-ligation may occur between adapters, target polynucleotide fragments, and/or barcodes. These self-ligations reduce the amount of starting material and decrease the yield of the desired product, for example, a polynucleotide fragment properly ligated to a barcode and/or and adapter.

This disclosure provides methods, compositions, systems, and devices for addressing this problem and increasing the yield of a desired product. The problem is addressed by pairing a first restriction enzyme and a second restriction enzyme. The two restriction enzymes create compatible termini upon cutting, but each enzyme has a different recognition sequence.

Ligation of two pieces of DNA generated after cutting with the first restriction enzyme will regenerate the recognition site for the first restriction enzyme, allowing the first restriction enzyme to re-cut the ligated DNA. Likewise, ligation of two pieces of DNA generated after cutting with the second restriction enzyme will regenerate the recognition site for the second restriction enzyme, allowing the second restriction enzyme to re-cut the ligated DNA. However, ligation of one piece of DNA generated after cutting with the first restriction enzyme and one piece of DNA generated after cutting with the second restriction enzyme will result in ligated DNA that is unrecognizable (and therefore uncuttable) by both the first and second enzymes. The result is that any multimers of fragmented target polynucleotides are re-cut and any multimers of adapter (or other molecules, e.g., barcodes) are also re-cut. However, when a fragmented target polynucleotide is properly ligated to an adapter (or barcode), the restriction sites for both enzymes are not present and the correctly ligated molecule may not be re-cut by either enzyme.

An example of this method is illustrated in FIG. **6**, and additional pairs of enzymes that may be used with the method are provided in FIGS. **7**A-**7**B. Any pair of enzymes may be used, so long as they meet the following criteria: (1) the enzymes should create identical, or at least similar, ligatable termini upon cutting; and (2) the enzymes should have different recognition sequences. The enzymes may be selected to avoid or minimize cutting of certain polynucleotide sequences such as barcodes, adapters, and other polynucleotide components of a sample processing or preparation platform. The enzymes may be selected for methylation insensitivity or methylation sensitivity. The enzymes may also be selected to be active under s single set of environmental conditions, such as buffer conditions, temperature, etc. Minimizing the cutting of barcodes and adapters may be accomplished by pairing certain enzymes with certain barcodes and/or adapters.

This method may be used to increase the yield of any of the barcoding methods described herein. The regeneration of the starting materials (e.g., fragmented target polynucleotide, adapters, and barcodes) allows these starting materials another opportunity to form the desired products (i.e., fragmented target polynucleotides ligated to barcodes, optionally with adapters). This greatly increases the yield of the reaction and therefore decreases the amount of starting material required to produce the necessary amount of the desired products while limiting the amount of undesirable side products and lost sequence information.

The methods described above may be used to achieve about 75%, 85%, 95%, 96%, 97%, 98%, 99%, or 99.5% yield (w/w). The methods may be used to achieve at least about 75%, 85%, 95%, 96%, 97%, 98%, 99%, or 99.5% yield (w/w).

The methods described above may use, for example, a pair of restriction enzyme selected from the group consisting of MspI-NarI, BfaI-NarI, BfaI-NdeI, HinP1I-ClaI, MseI-NdeI, CviQI-NdeI, TaqαI-AcII, RsaI-PmeI, AluI-EcoRV, BstUI-PmeI, DpnI-StuI, HaeIII-PmeI, and HpyCH4V-SfoI. This list of enzymes is provided for purposes of illustration only, and is not meant to be limiting.

The methods described above may generally use any two enzymes that create ligatable termini upon cutting but that have different recognition sequences. However, the method is not limited to ligation. For example, multimers formed after amplification of side products formed by association of compatible ends could also be re-cut using the methods described above.

More than one pair of enzymes may also be used. The number of pairs of enzymes chosen will vary depending on the number of undesirable side products formed in a reaction. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more pairs of enzymes may be used. Treatment of a polynucleotide with the enzymes may be sequential, simultaneous, or both.

V. Preparation of Target Polynucleotides

Target polynucleotides processed according to the methods provided in this disclosure may be DNA, RNA, peptide nucleic acids, and any hybrid thereof, where the polynucleotide contains any combination of deoxyribo- and ribonucleotides. Polynucleotides may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. Polynucleotides may contain any combination of nucleotides, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine and any nucleotide derivative thereof. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleoside and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species. Target polynucleotides may be cDNA, mitochondrial DNA (mtDNA), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear RNA (nRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), microRNA (miRNA), double stranded (dsRNA), ribozyme, riboswitch or viral RNA. Target polynucleotides may be contained on a plasmid, cosmid, or chromosome, and may be part of a genome. In some cases, a target polynucleotide may comprise one or more genes and/or one or more pseudogenes. A pseudogene generally refers to a dysfunctional relative of a gene that has lost its protein coding ability and/or is otherwise no longer expressed in the cell.

Target polynucleotides may be obtained from a sample using any methods known in the art. A target polynucleotide processed as described herein may be obtained from whole cells, cell preparations and cell-free compositions from any organism, tissue, cell, or environment. In some instances, target polynucleotides may be obtained from bodily fluids which may include blood, urine, serum, lymph, saliva, mucosal secretions, perspiration, or semen. In some instances, polynucleotides may be obtained from environmental samples including air, agricultural products, water, and soil. In other instances polynucleotides may be the products of experimental manipulation including, recombinant cloning, polynucleotide amplification (as generally described in PCT/US99/01705), polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

Genomic DNA may be obtained from naturally occurring or genetically modified organisms or from artificially or synthetically created genomes. Target polynucleotides comprising genomic DNA may be obtained from any source and using any methods known in the art. For example, genomic DNA may be isolated with or without amplification. Amplification may include PCR amplification, multiple displacement amplification (MDA), rolling circle amplification and other amplification methods. Genomic DNA may also be obtained by cloning or recombinant methods, such as those involving plasmids and artificial chromosomes or other conventional methods (see Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*., cited supra.) Polynucleotides may be isolated using other methods known in the art, for example as disclosed in *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) or *Molecular Cloning: A Laboratory Manual*. If the isolated polynucleotide is an mRNA, it may be reverse transcribed into cDNA using conventional techniques, as described in Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*., cited supra.

Target polynucleotides may also be isolated from "target organisms" or "target cells". The terms "target organism" and "target cell" refer to an organism or cell, respectively, from which target polynucleotides may be obtained. Target cells may be obtained from a variety of organisms including human, mammal, non-human mammal, ape, monkey, chimpanzee, plant, reptilian, amphibian, avian, fungal, viral or bacterial organisms. Target cells may also be obtained from a variety of clinical sources such as biopsies, aspirates, blood, urine, formalin fixed embedded tissues, and the like. Target cells may comprise a specific cell type, such as a somatic cell, germline cell, wild-type cell, cancer or tumor cells, or diseased or infected cell. A target cell may refer to

a cell derived from a particular tissue or a particular locus in a target organism. A target cell may comprise whole intact cells, or cell preparations.

Target polynucleotides may also be obtained or provided in specified quantities. Amplification may be used to increase the quantity of a target polynucleotide. Target polynucleotides may quantified by mass. For example, target polynucleotides may be provided in a mass ranging from about 1-10, 10-50, 50-100, 100-200, 200-1000, 1000-10000 ng. Target polynucleotides may be provided in a mass of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, or 10000 ng. Target polynucleotides may be provided in a mass of less than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, or 10000 ng.

Target polynucleotides may also be quantified as "genome equivalents." A genome equivalent is an amount of polynucleotide equivalent to one haploid genome of an organism from which the target polynucleotide is derived. For example, a single diploid cell contains two genome equivalents of DNA. Target polynucleotides may be provided in an amount ranging from about 1-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, or 100000-1000000 genome equivalents. Target polynucleotides may be provided in an amount of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, 20000, 30000, 40000, 50000, 60000 70000, 80000, 90000, 100000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, or 1000000 genome equivalents. Target polynucleotides may be provided in an amount less than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, 20000, 30000, 40000, 50000, 60000 70000, 80000, 90000, 100000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, or 1000000 genome equivalents.

Target polynucleotide may also be quantified by the amount of sequence coverage provided. The amount of sequence coverage refers to the average number of reads representing a given nucleotide in a reconstructed sequence. Generally, the greater the number of times a region is sequenced, the more accurate the sequence information obtained. Target polynucleotides may be provided in an amount that provides a range of sequence coverage from about 0.1×-10×, 10-×-50×, 50×-100×, 100×-200×, or 200×-500×. Target polynucleotide may be provided in an amount that provides at least about 0.1×, 0.2×, 0.3×, 0.4×, 0.5×, 0.6×, 0.7×, 0.8×, 0.9×, 1.0×, 5×, 10×, 25×, 50×, 100×, 125×, 150×, 175×, or 200× sequence coverage. Target polynucleotide may be provided in an amount that provides less than about 0.2×, 0.3×, 0.4×, 0.5×, 0.6×, 0.7×, 0.8×, 0.9×, 1.0×, 5×, 10×, 25×, 50×, 100×, 125×, 150×, 175×, or 200× sequence coverage.

VI. Fragmentation of Target Polynucleotides

Fragmentation of polynucleotides is used as a step in a variety of processing methods described herein. The size of the polynucleotide fragments, typically described in terms of length (quantified by the linear number of nucleotides per fragment), may vary depending on the source of the target polynucleotide, the method used for fragmentation, and the desired application. Moreover, while certain methods of the invention are illustrated using a certain number of fragmentation steps, the number of fragmentation steps provided is not meant to be limiting, and any number of fragmentation steps may be used. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more fragmentation steps may be used.

Fragments generated using the methods described herein may be about 1-10, 10-20, 20-50, 50-100, 50-200, 100-200, 200-300, 300-400, 400-500, 500-1000, 1000-5000, 5000-10000, 10000-100000, 100000-250000, or 250000-500000 nucleotides in length. Fragments generated using the methods described herein may be at least about 10, 20, 100, 200, 300, 400, 500, 1000, 5000, 10000, 100000, 250000, 500000, or more nucleotides in length. Fragments generated using the methods described herein may be less than about 10, 20, 100, 200, 300, 400, 500, 1000, 5000, 10000, 100000, 250000, 500000, nucleotides in length.

Fragments generated using the methods described herein may have a mean or median length of about 1-10, 10-20, 20-50, 50-100, 50-200, 100-200, 200-300, 300-400, 400-500, 500-1000, 1000-5000, 5000-10000, 10000-100000, 100000-250000, or 250000-500000 nucleotides. Fragments generated using the methods described herein may have a mean or median length of at least about 10, 20, 100, 200, 300, 400, 500, 1000, 5000, 10000, 100000, 250000, 500000, or more nucleotides. Fragments generated using the methods described herein may have a mean or median length of less than about 10, 20, 100, 200, 300, 400, 500, 1000, 5000, 10000, 100000, 250000, 500000, nucleotides.

Numerous fragmentation methods are described herein and known in the art. For example, fragmentation may be performed through physical, mechanical or enzymatic methods. Physical fragmentation may include exposing a target polynucleotide to heat or to UV light. Mechanical disruption may be used to mechanically shear a target polynucleotide into fragments of the desired range. Mechanical shearing may be accomplished through a number of methods known in the art, including repetitive pipetting of the target polynucleotide, sonication and nebulization. Target polynucleotides may also be fragmented using enzymatic methods. In some cases, enzymatic digestion may be performed using enzymes such as using restriction enzymes.

While the methods of fragmentation described in the preceding paragraph, and in some paragraphs of the disclosure, are described with reference to "target" polynucleotides, this is not meant to be limiting, above or anywhere else in this disclosure. Any means of fragmentation described herein, or known in the art, can be applied to any polynucleotide used with the invention. In some cases, this polynucleotide may be a target polynucleotide, such as a genome. In other cases, this polynucleotide may be a fragment of a target polynucleotide which one wishes to further fragment. In still other cases, still further fragments may be still further fragmented. Any suitable polynucleotide may be fragmented according the methods described herein.

A fragment of a polynucleotide generally comprises a portion of the sequence of the targeted polynucleotide from which the fragment was generated. In some cases, a fragment may comprise a copy of a gene and/or pseudogene, including one included in the original target polynucleotide. In some cases, a plurality of fragments generated from fragmenting a target polynucleotide may comprise fragments that each comprise a copy of a gene and/or pseudogene.

Restriction enzymes may be used to perform specific or non-specific fragmentation of target polynucleotides. The methods of the present disclosure may use one or more types

of restriction enzymes, generally described as Type I enzymes, Type II enzymes, and/or Type III enzymes. Type II and Type III enzymes are generally commercially available and well known in the art. Type II and Type III enzymes recognize specific sequences of nucleotide base pairs within a double stranded polynucleotide sequence (a "recognition sequence" or "recognition site"). Upon binding and recognition of these sequences, Type II and Type III enzymes cleave the polynucleotide sequence. In some cases, cleavage will result in a polynucleotide fragment with a portion of overhanging single stranded DNA, called a "sticky end." In other cases, cleavage will not result in a fragment with an overhang, creating a "blunt end." The methods of the present disclosure may comprise use of restriction enzymes that generate either sticky ends or blunt ends.

Restriction enzymes may recognize a variety of recognition sites in the target polynucleotide. Some restriction enzymes ("exact cutters") recognize only a single recognition site (e.g., GAATTC). Other restriction enzymes are more promiscuous, and recognize more than one recognition site, or a variety of recognition sites. Some enzymes cut at a single position within the recognition site, while others may cut at multiple positions. Some enzymes cut at the same position within the recognition site, while others cut at variable positions.

The present disclosure provides method of selecting one or more restriction enzymes to produce fragments of a desired length. Polynucleotide fragmentation may be simulated in silico, and the fragmentation may be optimized to obtain the greatest number or fraction of polynucleotide fragments within a particular size range, while minimizing the number or fraction of fragments within undesirable size ranges. Optimization algorithms may be applied to select a combination of two or more enzymes to produce the desired fragment sizes with the desired distribution of fragments quantities.

A polynucleotide may be exposed to two or more restriction enzymes simultaneously or sequentially. This may be accomplished by, for example, adding more than one restriction enzyme to a partition, or by adding one restriction enzyme to a partition, performing the digestion, deactivating the restriction enzyme (e.g., by heat treatment) and then adding a second restriction enzyme. Any suitable restriction enzyme may be used alone, or in combination, in the methods presented herein.

Fragmenting of a target polynucleotide may occur prior to partitioning of the target polynucleotide or fragments generated from fragmenting. For example, genomic DNA (gDNA) may be fragmented, using, for example, a restriction enzyme, prior to the partitioning of its generated fragments. In another example, a target polynucleotide may be entered into a partition along with reagents necessary for fragmentation (e.g., including a restriction enzyme), such that fragmentation of the target polynucleotide occurs within the partition. For example, gDNA may be fragmented in a partition comprising a restriction enzyme, and the restriction enzyme is used to fragment the gDNA.

In some cases, a plurality of fragments may be generated prior to partitioning, using any method for fragmentation described herein. Some or all of the fragments of the plurality, for example, may each comprise a copy of a gene and/or a pseudogene. The fragments can be separated and partitioned such that each copy of the gene or pseudogene is located in a different partition. Each partition, for example, can comprise a different barcode sequence such that each copy of the gene and/or pseudogene can be associated with a different barcode sequence, using barcoding methods described elsewhere herein. Via the different barcode sequences, each gene and/or pseudogene can be counted and/or differentiated during sequencing of the barcoded fragments. Any sequencing method may be used, including those described herein.

For example, using restriction enzymes, genomic DNA (gDNA) can be fragmented to generate a plurality of non-overlapping fragments of the gDNA. At least some of the fragments of the plurality may each comprise a copy of a gene and/or a pseudogene. The fragments may be separated and partitioned such that each copy of the gene or pseudogene is located in a different partition. Each partition, for example, can comprise a different barcode sequence such that each copy of the gene and/or pseudogene may be barcoded with a different barcode sequence. Via the different barcode sequences, the genes and/or pseudogenes may be counted and or differentiated after sequencing of the barcoded fragments. Any sequencing method may be used, including those described herein.

IV. Partitioning of Polynucleotides

As described throughout the disclosure, certain methods, systems, and compositions of the disclosure may utilize partitioning of polynucleotides into separate partitions (e.g., microwells, droplets of an emulsion). These partitions may be used to contain polynucleotides for further processing, such as, for example, cutting, ligating, and/or barcoding.

Any number of devices, systems or containers may be used to hold, support or contain partitions of polynucleotides and their fragments. In some cases, partitions are formed from droplets, emulsions, or spots on a substrate. Weizmann et al. (*Nature Methods,* 2006, Vol. 3 No. 7 pages 545-550). Suitable methods for forming emulsions, which can be used as partitions or to generate microcapsules, include the methods described in Weitz et al. (U.S. Pub. No. 2012/0211084). Partitions may also be formed through the use of wells, microwells, multi-well plates, and microwell arrays. Partitioning may be performed using piezoelectric droplet generation (e.g., Bransky et al., *Lab on a Chip,* 2009, 9, 516-520). Partitioning may be performed using surface acoustic waves (e.g., Demirci and Montesano, *Lab on a Chip,* 2007, 7, 1139-1145).

Each partition may also contain, or be contained within any other suitable partition. For example, a well, microwell, hole, a surface of a bead, or a tube may comprise a droplet (e.g., a droplet in an emulsion), a continuous phase in an emulsion, a spot, a capsule, or any other suitable partition. A droplet may comprise a capsule, bead, or another droplet. A capsule may comprise a droplet, bead, or another capsule. These descriptions are merely illustrative, and all suitable combinations and pluralities are also envisioned. For example, any suitable partition may comprise a plurality of the same or different partitions. In one example, a well or microwell comprises a plurality of droplets and a plurality of capsules. In another example, a capsule comprises a plurality of capsules and a plurality of droplets. All combinations of partitions are envisioned. Table 1 shows non-limiting examples of partitions that may be combined with each other.

TABLE 1

Examples of partitions that may be combined with each other.

	Well	Spot	Droplet	Capsule
Well	Well inside well	Spot inside well	Droplet inside well	Capsule inside well

TABLE 1-continued

Examples of partitions that may be combined with each other.

	Well	Spot	Droplet	Capsule
Spot	Spot inside well	Spot inside spot	Droplet inside spot	Capsule inside spot
Droplet	Droplet inside well	Droplet inside spot	Droplet inside droplet	Droplet inside capsule Capsule inside droplet
Capsule	Capsule inside well	Capsule inside spot Spot inside capsule	Capsule inside droplet Droplet inside capsule	Capsule inside capsule
Surface of a Bead	Bead inside well	Spot on bead Bead inside spot	Bead inside droplet	Bead inside capsule

Any partition described herein may comprise multiple partitions. For example, a partition may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 partitions. A partition may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 partitions. In some cases, a partition may comprise less than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, or 50000 partitions. In some cases, each partition may comprise 2-50, 2-20, 2-10, or 2-5 partitions.

The number of partitions employed may vary depending on the application. For example, the number of partitions may be about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, or 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100,000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1,000,000, 2,000,000, 3,000,000, 4,000,000, 5,000,000, 10000000, 20000000, or more. The number of partitions may be at least about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100,000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1,000,000, 2,000,000, 3,000,000, 4,000,000, 5,000,000, 10000000, 20000000, or more. The number of partitions may be less than about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100,000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1,000,000, 2,000,000, 3,000,000, 4,000,000, 5,000,000, 10000000, 20000000. The number of partitions may be about 5-10000000, 5-5000000, 5-1,000,000, 10-10,000, 10-5,000, 10-1,000, 1,000-6,000, 1,000-5,000, 1,000-4,000, 1,000-3,000, or 1,000-2,000.

Such partitions may be pre-loaded with reagents to perform a particular reaction. For example, a capsule containing one or more reagents may be placed within a microwell. After adding a polynucleotide sample to the well, the capsule may be made to release its contents. The contents of the capsule may include, for example, restriction enzymes, ligases, barcodes, and adapters for processing the polynucleotide sample placed in the microwell.

In some cases, such partitions may be droplets of an emulsion. For example, a droplet of an emulsion may be an aqueous droplet in an oil phase. The droplet may comprise, for example, one or more reagents (e.g., restriction enzymes, ligases, polymerases, reagents necessary for nucleic acid amplification (e.g., primers, DNA polymerases, dNTPs, buffers)), a polynucleotide sample, and a barcode sequence. In some cases, the barcode sequence, polynucleotide sample, or any reagent may be associated with a solid surface within a droplet. In some cases, the solid surface is a bead. In some cases, the bead is a gel bead (see e.g., Agresti et al., U.S. Patent Publication No. 2010/0136544). In some cases the droplet is hardened into a gel bead (e.g., via polymerization).

A species may be contained within a droplet in an emulsion containing, for example, a first phase (e.g., oil or water) forming the droplet and a second (continuous) phase (e.g., water or oil). An emulsion may be a single emulsion, for example, a water-in-oil or an oil-in-water emulsion. An emulsion may be a double emulsion, for example a water-in-oil-in-water or an oil-in-water-in-oil emulsion. Higher-order emulsions are also possible. The emulsion may be held in any suitable container, including any suitable partition described in this disclosure.

In some cases, droplets in an emulsion comprise other partitions. A droplet in an emulsion may comprise any suitable partition including, for example, another droplet (e.g., a droplet in an emulsion), a capsule, a bead, and the like. Each partition may be present as a single partition or a plurality of partitions, and each partition may comprise the same species or different species.

In one example, a droplet in an emulsion comprises a capsule comprising reagents for sample processing. As described elsewhere in this disclosure, a capsule may contain one or more capsules, or other partitions. A sample comprising an analyte to be processed is contained within the droplet. A stimulus is applied to cause release of the contents of the capsule into the droplet, resulting in contact between the reagents and the analyte to be processed. The droplet is incubated under appropriate conditions for the processing of the analyte. Processed analyte may then be recovered. While this example describes an embodiment where a reagent is in a capsule and an analyte is in the droplet, the opposite configuration—i.e., reagent in the droplet and analyte in the capsule—is also possible.

The droplets in an emulsion may be of uniform size or heterogeneous size. In some cases, the diameter of a droplet in an emulsion may be about 0.001 μm, 0.01 μm, 0.05 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 150 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, or 1 mm. A droplet may have a diameter of at least about 0.001 μm, 0.01 μm, 0.05 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 150 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, or 1 mm. In some cases, a droplet may have a diameter of less than about 0.001 μm, 0.01 μm, 0.05 μm, 0.1 μm, 0.5 μm, 1 μm, 5 μm, 10 μm, 50 μm, 100 μm, 150 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, or 1 mm. In some cases, a droplet may have a diameter of about 0.001 μm to 1 mm, 0.01 μm to 900 μm, 0.1 μm to 600 μm, 100 μm to 200 μm, 100 μm to 300 μm, 100 μm to 400 μm, 100 μm to 500 μm, 100 μm to 600 μm, 150 μm to 200 μm, 150 μm to 300 μm, or 150 μm to 400 μm.

Droplets in an emulsion also may have a particular density. In some cases, the droplets are less dense than an aqueous fluid (e.g., water); in some cases, the droplets are denser than an aqueous fluid. In some cases, the droplets are less dense than a non-aqueous fluid (e.g., oil); in some cases,

the droplets are denser than a non-aqueous fluid. Droplets may have a density of about 0.05 g/cm^3, 0.1 g/cm^3, 0.2 g/cm^3, 0.3 g/cm^3, 0.4 g/cm^3, 0.5 g/cm^3, 0.6 g/cm^3, 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. Droplets may have a density of at least about 0.05 g/cm^3, 0.1 g/cm^3, 0.2 g/cm^3, 0.3 g/cm^3, 0.4 g/cm^3, 0.5 g/cm^3, 0.6 g/cm^3, 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. In other cases, droplet densities may be at most about 0.7 g/cm^3, 0.8 g/cm^3, 0.81 g/cm^3, 0.82 g/cm^3, 0.83 g/cm^3, 0.84 g/cm^3, 0.85 g/cm^3, 0.86 g/cm^3, 0.87 g/cm^3, 0.88 g/cm^3, 0.89 g/cm^3, 0.90 g/cm^3, 0.91 g/cm^3, 0.92 g/cm^3, 0.93 g/cm^3, 0.94 g/cm^3, 0.95 g/cm^3, 0.96 g/cm^3, 0.97 g/cm^3, 0.98 g/cm^3, 0.99 g/cm^3, 1.00 g/cm^3, 1.05 g/cm^3, 1.1 g/cm^3, 1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3, 1.5 g/cm^3, 1.6 g/cm^3, 1.7 g/cm^3, 1.8 g/cm^3, 1.9 g/cm^3, 2.0 g/cm^3, 2.1 g/cm^3, 2.2 g/cm^3, 2.3 g/cm^3, 2.4 g/cm^3, or 2.5 g/cm^3. Such densities can reflect the density of the capsule in any particular fluid (e.g., aqueous, water, oil, etc.)

Polynucleotides may be partitioned using a variety of methods. For example, polynucleotides may be diluted and dispensed across a plurality of partitions. A terminal dilution of a medium comprising polynucleotides may be performed such that the number of partitions or wells exceeds the number of polynucleotides. The ratio of the number of polynucleotides to the number of partitions may range from about 0.1-10, 0.5-10, 1-10, 2-10, 10-100, 100-1000, or more. The ratio of the number of polynucleotides to the number of partitions may be about 0.1, 0.5, 1, 2, 4, 8, 10, 20, 50, 100, or 1000. The ratio of the number of polynucleotides to the number of partitions may be at least about 0.1, 0.5, 1, 2, 4, 8, 10, 20, 50, 100, or 1000. The ratio of the number of polynucleotides to the number of partitions may be less than about 0.1, 0.5, 1, 2, 4, 8, 10, 20, 50, 100, or 1000.

The number of partitions employed may vary depending on the application. For example, the number of partitions may be about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, or 10,000, or more. The number of partitions may be at least about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, or 10,000, or more. The number of partitions may be less than about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, or 10,000.

The volume of the partitions may vary depending on the application. For example, the volume of the partitions may be about 1000 μl, 900 μl, 800 μl, 700 μl, 600 μl, 500 μl, 400 μl, 300 μl, 200 μl, 100 μl, 50 μl, 25 μl, 10 μl, 5 μl, 1 μl, 900 nL, 800 nL, 700 nL, 600 nL, 500 nL, 400 nL, 300 nL, 200 nL, 100 nL, 50 nL, 25 nL, 10 nL, or 5 nL. The volume of the partitions may be at least about 1000 μl, 900 μl, 800 μl, 700 μl, 600 μl, 500 μl, 400 μl, 300 μl, 200 μl, 100 μl, 50 μl, 25 μl, 10 μl, 5 μl, 1 μl, 900 nL, 800 nL, 700 nL, 600 nL, 500 nL, 400 nL, 300 nL, 200 nL, 100 nL, 50 nL, 25 nL, 10 nL, or 5 nL. The volume of the partitions may be less than about 1000 μl, 900 μl, 800 μl, 700 μl, 600 μl, 500 μl, 400 μl, 300 μl, 200 μl, 100 μl, 50 μl, 25 μl, 10 μl, 5 μl, 1 μl, 900 nL, 800 nL, 700 nL, 600 nL, 500 nL, 400 nL, 300 nL, 200 nL, 100 nL, 50 nL, 25 nL, 10 nL, or 5 nL.

Species may also be partitioned at a particular density. For example, species may be partitioned so that each partition contains about 1, 5, 10, 50, 100, 1000, 10000, 100000, or 1000000 species per partition. Species may be partitioned so that each partition contains at least about 1, 5, 10, 50, 100, 1000, 10000, 100000, 1000000 or more species per partition. Species may be partitioned so that each partition contains less than about 1, 5, 10, 50, 100, 1000, 10000, 100000, or 1000000 species per partition. Species may be partitioned such that each partition contains about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, or 100000-1000000 species per partition.

Species may be partitioned such that at least one partition comprises a species that is unique within that partition. This may be true for about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of the partitions. This may be true for at least about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of the partitions. This may be true for less than about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the partitions.

Particular polynucleotides may also be targeted to specific partitions. For example, in some cases, a capture reagent such as an oligonucleotide probe may be immobilized in a partition to capture specific polynucleotides through hybridization.

Polynucleotides may also be partitioned at a particular density. For example, polynucleotides may be partitioned such that each partition contains about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, or 100000-1000000 polynucleotides per partition. Polynucleotides may be partitioned so that each partition contains about 1, 5, 10, 50, 100, 1000, 10000, 100000, 1000000 or more polynucleotides per partition. Polynucleotides may be partitioned so that each partition contains less than about 1, 5, 10, 50, 100, 1000, 10000, 100000, or 1000000 polynucleotides per partition. Polynucleotides may be partitioned so that each partition contains at least about 1, 5, 10, 50, 100, 1000, 10000, 100000, or 1000000 polynucleotides per partition.

Polynucleotides may be partitioned such that at least one partition comprises a polynucleotide sequence with a unique sequence compared to all other polynucleotide sequences contained within the same partition. This may be true for about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of the partitions. This may be true for less than about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of the partitions. This may be true for more than about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of the partitions.

V. Barcoding

Downstream applications, for example DNA sequencing, may rely on the barcodes to identify the origin of a sequence and, for example, to assemble a larger sequence from sequenced fragments. Therefore, it may be desirable to add barcodes to the polynucleotide fragments generated by the methods described herein. Barcodes may be of a variety of different formats, including polynucleotide barcodes. Depending upon the specific application, barcodes may be attached to polynucleotide fragments in a reversible or irreversible manner. Barcodes may also allow for identification and/or quantification of individual polynucleotide fragments during sequencing.

Barcodes may be loaded into partitions so that one or more barcodes are introduced into a particular partition. Each partition may contain a different set of barcodes. This

may be accomplished by directly dispensing the barcodes into the partitions, enveloping the barcodes (e.g., in a droplet of an emulsion), or by placing the barcodes within a container that is placed in a partition (e.g., a microcapsule).

The number of partitions employed may vary depending on the application. For example, the number of partitions may be about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, or 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100,000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1,000,000, 2,000,000, 3,000,000, 4,000,000, 5,000,000, 10000000, 20000000, or more. The number of partitions may be at least about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100,000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1,000,000, 2,000,000, 3,000,000, 4,000,000, 5,000,000, 10000000, 20000000, or more. The number of partitions may be less than about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100,000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1,000,000, 2,000,000, 3,000,000, 4,000,000, 5,000,000, 10000000, 20000000. The number of partitions may be about 5-10000000, 5-5000000, 5-1,000,000, 10-10,000, 10-5,000, 10-1,000, 1,000-6,000, 1,000-5,000, 1,000-4,000, 1,000-3,000, or 1,000-2,000.

The number of different barcodes or different sets of barcodes that are partitioned may vary depending upon, for example, the particular barcodes to be partitioned and/or the application. Different sets of barcodes may be, for example, sets of identical barcodes where the identical barcodes differ between each set. Or different sets of barcodes may be, for example, sets of different barcodes, where each set differs in its included barcodes. For example, about 1, 5, 10, 50, 100, 1000, 10000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, 100000000, or more different barcodes or different sets of barcodes may be partitioned. In some examples, at least about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, 100000000, or more different barcodes or different sets of barcodes may be partitioned. In some examples, less than about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 different barcodes or different sets of barcodes may be partitioned. In some examples, about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, 100000-1000000, 10000-1000000, 10000-10000000, or 10000-100000000 barcodes may be partitioned.

Barcodes may be partitioned at a particular density. For example, barcodes may be partitioned so that each partition contains about 1, 5, 10, 50, 100, 1000, 10000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 barcodes per partition. Barcodes may be partitioned so that each partition contains at least about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 barcodes per partition. Barcodes may be partitioned so that each partition contains less than about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 barcodes per partition. Barcodes may be partitioned such that each partition contains about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, 100000-1000000, 10000-1000000, 10000-10000000, or 10000-100000000 barcodes per partition.

Barcodes may be partitioned such that identical barcodes are partitioned at a particular density. For example, identical barcodes may be partitioned so that each partition contains about 1, 5, 10, 50, 100, 1000, 10000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 identical barcodes per partition. Barcodes may be partitioned so that each partition contains at least about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, 100000000, or more identical barcodes per partition. Barcodes may be partitioned so that each partition contains less than about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 identical barcodes per partition. Barcodes may be partitioned such that each partition contains about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, 100000-1000000, 10000-1000000, 10000-10000000, or 10000-100000000 identical barcodes per partition.

Barcodes may be partitioned such that different barcodes are partitioned at a particular density. For example, different barcodes may be partitioned so that each partition contains about 1, 5, 10, 50, 100, 1000, 10000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 different barcodes per partition. Barcodes may be partitioned so that each partition contains at least about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, 100000000, or more different barcodes per partition. Barcodes may be partitioned so that each partition contains less than about 1, 5, 10, 50, 100, 1000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000,

90000, 100000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000, 10000000, 20000000, 50000000, or 100000000 different barcodes per partition. Barcodes may be partitioned such that each partition contains about 1-5, 5-10, 10-50, 50-100, 100-1000, 1000-10000, 10000-100000, 100000-1000000, 10000-1000000, 10000-10000000, or 10000-100000000 different barcodes per partition.

The number of partitions employed to partition barcodes may vary, for example, depending on the application and/or the number of different barcodes to be partitioned. For example, the number of partitions employed to partition barcodes may be about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, or 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100,000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1,000,000, 2,000,000, 3,000,000, 4,000,000, 5,000,000, 10000000, 20000000 or more. The number of partitions employed to partition barcodes may be at least about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1000000, 2000000, 3000000, 4000000, 5000000, 10000000, 20000000 or more. The number of partitions employed to partition barcodes may be less than about 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500, 5000, 7500, 10,000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1000000, 2000000, 3000000, 4000000, 5000000, 10000000, or 20000000. The number of partitions employed to partition barcodes may be about 5-10000000, 5-5000000, 5-1,000,000, 10-10,000, 10-5,000, 10-1,000, 1,000-6,000, 1,000-5,000, 1,000-4,000, 1,000-3,000, or 1,000-2,000. As described above, different barcodes or different sets of barcodes (e.g., each set comprising a plurality of identical barcodes or different barcodes) may be partitioned such that each partition comprises a different barcode or different barcode set. In some cases, each partition may comprise a different set of identical barcodes. Where different sets of identical barcodes are partitioned, the number of identical barcodes per partition may vary. For example, about 100,000 or more different sets of identical barcodes may be partitioned across about 100,000 or more different partitions, such that each partition comprises a different set of identical barcodes. In each partition, the number of identical barcodes per set of barcodes may be about 1,000,000 identical barcodes. In some cases, the number of different sets of barcodes may be equal to or substantially equal to the number of partitions. Any suitable number of different barcodes or different barcode sets (including numbers of different barcodes or different barcode sets to be partitioned described elsewhere herein), number of barcodes per partition (including numbers of barcodes per partition described elsewhere herein), and number of partitions (including numbers of partitions described elsewhere herein) may be combined to generate a diverse library of partitioned barcodes with high numbers of barcodes per partition. Thus, as will be appreciated, any of the above-described different numbers of barcodes may be provided with any of the above-described barcode densities per partition, and in any of the above-described numbers of partitions.

For example, a population of microcapsules may be prepared such that a first microcapsule in the population comprises multiple copies of identical barcodes (e.g., polynucleotide bar codes, etc.) and a second microcapsule in the population comprises multiple copies of a barcode that differs from the barcode within the first microcapsule. In some cases, the population of microcapsules may comprise multiple microcapsules (e.g., greater than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules), each containing multiple copies of a barcode that differs from that contained in the other microcapsules. In some cases, the population may comprise greater than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules with identical sets of barcodes. In some cases, the population may comprise greater than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 500, 1000, 5000, 10000, 100000, 1000000, 10000000, 100000000, or 1000000000 microcapsules, wherein the microcapsules each comprise a different combination of barcodes. For example, in some cases the different combinations overlap, such that a first microcapsule may comprise, e.g., barcodes A, B, and C, while a second microcapsule may comprise barcodes A, B, and D. In another example, the different combinations do not overlap, such that a first microcapsule may comprise, e.g., barcodes A, B, and C, while a second microcapsule may comprise barcodes D, E, and F. The use of microcapsules is, of course, optional. All of the combinations described above, and throughout this disclosure, may also be generated by dispending barcodes (and other reagents) directly into partitions (e.g., microwells).

The barcodes may be loaded into the partitions at an expected or predicted ratio of barcodes per species to be barcoded (e.g., polynucleotide fragment, strand of polynucleotide, cell, etc.). In some cases, the barcodes are loaded into partitions such that more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 barcodes are loaded per species. In some cases, the barcodes are loaded in the partitions so that less than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 barcodes are loaded per species. In some cases, the average number of barcodes loaded per species is less than, or greater than, about 0.0001, 0.001, 0.01, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 200000 barcodes per species.

When more than one barcode is present per polynucleotide fragment, such barcodes may be copies of the same barcode, or multiple different barcodes. For example, the attachment process may be designed to attach multiple identical barcodes to a single polynucleotide fragment, or multiple different barcodes to the polynucleotide fragment.

The methods provided herein may comprise loading a partition (e.g., a microwell, droplet of an emulsion) with the reagents necessary for the attachment of barcodes to polynucleotide fragments. In the case of ligation reactions, reagents including restriction enzymes, ligase enzymes, buffers, adapters, barcodes and the like may be loaded into a partition. In the case barcoding by amplification, reagents including primers, DNA polymerases, DNTPs, buffers, barcodes and the like may be loaded into a partition. As described throughout this disclosure, these reagents may be loaded directly into the partition, or via a container such as a microcapsule. If the reagents are not disposed within a container, they may be loaded into a partition (e.g., a microwell) which may then be sealed with a wax or oil until the reagents are used.

Barcodes may be ligated to a polynucleotide fragment using sticky or blunt ends. Barcoded polynucleotide frag-

ments may also be generated by amplifying a polynucleotide fragment with primers comprising barcodes.

Barcodes may be assembled combinatorially, from smaller components designed to assemble in a modular format. For example, three modules, 1A, 1B, and 1C may be combinatorially assembled to produce barcode 1ABC. Such combinatorial assembly may significantly reduce the cost of synthesizing a plurality of barcodes. For example, a combinatorial system consisting of 3 A modules, 3 B modules, and 3 C modules may generate 3*3*3=27 possible barcode sequences from only 9 modules.

VI. Microcapsules and Microwell Capsule Arrays

Microcapsules and microwell capsule array (MCA) devices may be used to perform the polynucleotide processing methods described herein. MCA devices are devices with a plurality of microwells. Microcapsules are introduced into these microwells, before, after, or concurrently with the introduction of a sample.

Microwells may comprise free reagents and/or reagents encapsulated in microcapsules. Any of the reagents described in this disclosure may be encapsulated in a microcapsule, including any chemicals, particles, and elements suitable for sample processing reactions involving a polynucleotide. For example, a microcapsule used in a sample preparation reaction for DNA sequencing may comprise one or more of the following reagents: enzymes, restriction enzymes (e.g., multiple cutters), ligase, polymerase, fluorophores, oligonucleotide barcodes, adapters, buffers, dNTPs, ddNTPs and the like.

Additional exemplary reagents include: buffers, acidic solution, basic solution, temperature-sensitive enzymes, pH-sensitive enzymes, light-sensitive enzymes, metals, metal ions, magnesium chloride, sodium chloride, manganese, aqueous buffer, mild buffer, ionic buffer, inhibitor, enzyme, protein, polynucleotide, antibodies, saccharides, lipid, oil, salt, ion, detergents, ionic detergents, non-ionic detergents, oligonucleotides, nucleotides, deoxyribonucleotide triphosphates (dNTPs), dideoxyribonucleotide triphosphates (ddNTPs), DNA, RNA, peptide polynucleotides, complementary DNA (cDNA), double stranded DNA (dsDNA), single stranded DNA (ssDNA), plasmid DNA, cosmid DNA, chromosomal DNA, genomic DNA, viral DNA, bacterial DNA, mtDNA (mitochondrial DNA), mRNA, rRNA, tRNA, nRNA, siRNA, snRNA, snoRNA, scaRNA, microRNA, dsRNA, ribozyme, riboswitch and viral RNA, polymerase, ligase, restriction enzymes, proteases, nucleases, protease inhibitors, nuclease inhibitors, chelating agents, reducing agents, oxidizing agents, fluorophores, probes, chromophores, dyes, organics, emulsifiers, surfactants, stabilizers, polymers, water, small molecules, pharmaceuticals, radioactive molecules, preservatives, antibiotics, aptamers, and pharmaceutical drug compounds.

In some cases, a microcapsule comprises a set of reagents that have a similar attribute (e.g., a set of enzymes, a set of minerals, a set of oligonucleotides, a mixture of different bar-codes, a mixture of identical bar-codes). In other cases, a microcapsule comprises a heterogeneous mixture of reagents. In some cases, the heterogeneous mixture of reagents comprises all components necessary to perform a reaction. In some cases, such mixture comprises all components necessary to perform a reaction, except for 1, 2, 3, 4, 5, or more components necessary to perform a reaction. In some cases, such additional components are contained within a different microcapsule or within a solution within a partition (e.g., microwell) of the device.

In some cases, only microcapsules comprising reagents are introduced. In other cases, both free reagents and reagents encapsulated in microcapsules are loaded into the device, either sequentially or concurrently. In some cases, reagents are introduced to the device either before or after a particular step. In some cases, reagents and/or microcapsules comprising reagents are introduced sequentially such that different reactions or operations occur at different steps. The reagents (or microcapsules) may be also be loaded at steps interspersed with a reaction or operation step. For example, microcapsules comprising reagents for fragmenting polynucleotides (e.g., restriction enzymes) may be loaded into the device, followed by loading of microcapsules comprising reagents for ligating bar-codes and subsequent ligation of the bar-codes to the fragmented molecules.

Microcapsules may be pre-formed and filled with reagents by injection. For example, the picoinjection methods described in Abate et al. (Proc. Natl. Acad. Sci. U.S.A., 2010, 107(45), 19163-19166) and Weitz et al. (U.S. Pub. No. 2012/0132288) may be used to introduce reagents into the interior of microcapsules described herein. These methods can also be used to introduce a plurality of any of the reagents described herein into microcapsules.

Microcapsules may be formed by any emulsion technique known in the art. For example, the multiple emulsion technique of Weitz et al. (U.S. Pub. No. 2012/0211084) may be used to form microcapsules (or partitions) for use with the methods disclosed herein.

Numerous chemical triggers may be used to trigger the disruption of partitions (e.g., Plunkett et al., Biomacromolecules, 2005, 6:632-637). Examples of these chemical changes may include, but are not limited to pH-mediated changes to the integrity of a component of a partition, disintegration of a component of a partition via chemical cleavage of crosslink bonds, and triggered depolymerization of a component of a partition. Bulk changes may also be used to trigger disruption of partitions.

A change in pH of a solution, such as a decrease in pH, may trigger disruption of a partition via a number of different mechanisms. The addition of acid may cause degradation or disassembly a portion of a partition through a variety of mechanisms. Addition of protons may disassemble cross-linking of polymers in a component of a partition, disrupt ionic or hydrogen bonds in a component of a partition, or create nanopores in a component of a partition to allow the inner contents to leak through to the exterior. A change in pH may also destabilize an emulsion, leading to release of the contents of the droplets.

In some examples, a partition is produced from materials that comprise acid-degradable chemical cross-linkers, such a ketals. A decrease in pH, particular to a pH lower than 5, may induce the ketal to convert to a ketone and two alcohols and facilitate disruption of the partition. In other examples, the partitions may be produced from materials comprising one or more polyelectrolytes that are pH sensitive. A decrease in pH may disrupt the ionic- or hydrogen-bonding interactions of such partitions, or create nanopores therein. In some cases, partitions made from materials comprising polyelectrolytes comprise a charged, gel-based core that expands and contracts upon a change of pH.

Disruption of cross-linked materials comprising a partition can be accomplished through a number of mechanisms. In some examples, a partition can be contacted with various chemicals that induce oxidation, reduction or other chemical changes. In some cases, a reducing agent, such as beta-mercaptoethanol, can be used, such that disulfide bonds of a partition are disrupted. In addition, enzymes may be added to cleave peptide bonds in materials forming a partition, thereby resulting in a loss of integrity of the partition.

Depolymerization can also be used to disrupt partitions. A chemical trigger may be added to facilitate the removal of a protecting head group. For example, the trigger may cause removal of a head group of a carbonate ester or carbamate within a polymer, which in turn causes depolymerization and release of species from the inside of a partition.

In yet another example, a chemical trigger may comprise an osmotic trigger, whereby a change in ion or solute concentration in a solution induces swelling of a material used to make a partition. Swelling may cause a buildup of internal pressure such that a partition ruptures to release its contents. Swelling may also cause an increase in the pore size of the material, allowing species contained within the partition to diffuse out, and vice versa.

A partition may also be made to release its contents via bulk or physical changes, such as pressure induced rupture, melting, or changes in porosity.

VII. Polynucleotide Sequencing

Generally, the methods and compositions provided herein are useful for preparation of polynucleotide fragments for downstream applications such as sequencing. Sequencing may be performed by any available technique. For example, sequencing may be performed by the classic Sanger sequencing method. Sequencing methods may also include: high-throughput sequencing, pyrosequencing, sequencing-by-synthesis, single-molecule sequencing, nanopore sequencing, sequencing-by-ligation, sequencing-by-hybridization, RNA-Seq (Illumina), Digital Gene Expression (Helicos), next generation sequencing, single molecule sequencing by synthesis (SMSS) (Helicos), massively-parallel sequencing, clonal single molecule Array (Solexa), shotgun sequencing, Maxim-Gilbert sequencing, primer walking, and any other sequencing methods known in the art.

In some cases varying numbers of fragments are sequenced. For example, in some cases about 30%-90% of the fragments are sequenced. In some cases, about 35%-85%, 40%-80%, 45%-75%, 50%-70%, 55%-65%, or 50%-60% of the fragments are sequenced. In some cases, at least about 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the fragments are sequenced. In some cases less than about 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the fragments are sequenced.

In some cases sequences from fragments are assembled to provide sequence information for a contiguous region of the original target polynucleotide that is longer than the individual sequence reads. Individual sequence reads may be about 10-50, 50-100, 100-200, 200-300, 300-400, or more nucleotides in length.

The identities of the barcode tags may serve to order the sequence reads from individual fragments as well as to differentiate between haplotypes. For example, during the partitioning of individual fragments, parental polynucleotide fragments may separated into different partitions. With an increase in the number of partitions, the likelihood of a fragment from both a maternal and paternal haplotype contained in the same partition becomes negligibly small. Thus, sequence reads from fragments in the same partition may be assembled and ordered.

VIII. Polynucleotide Phasing

This disclosure also provides methods and compositions to prepare polynucleotide fragments in such a manner that may enable phasing or linkage information to be generated. Such information may allow for the detection of linked genetic variations in sequences, including genetic variations (e.g., SNPs, mutations, indels, copy number variations, transversions, translocations, inversions, etc.) that are separated by long stretches of polynucleotides. The term "indel" refers to a mutation resulting in a colocalized insertion and deletion and a net gain or loss in nucleotides. A "microindel" is an indel that results in a net gain or loss of 1 to 50 nucleotides. These variations may exist in either a cis or trans relationship. In a cis relationship, two or more genetic variations exist in the same polynucleotide or strand. In a trans relationship, two or more genetic variations exist on multiple polynucleotide molecules or strands.

Methods provided herein may be used to determine polynucleotide phasing. For example, a polynucleotide sample (e.g., a polynucleotide that spans a given locus or loci) may be partitioned such that at most one molecule of polynucleotide is present per partition (e.g., microwell). The polynucleotide may then be fragmented, barcoded, and sequenced. The sequences may be examined for genetic variation. The detection of genetic variations in the same sequence tagged with two different bar codes may indicate that the two genetic variations are derived from two separate strands of DNA, reflecting a trans relationship. Conversely, the detection of two different genetic variations tagged with the same bar codes may indicate that the two genetic variations are from the same strand of DNA, reflecting a cis relationship.

Phase information may be important for the characterization of a polynucleotide fragment, particularly if the polynucleotide fragment is derived from a subject at risk of, having, or suspected of a having a particular disease or disorder (e.g., hereditary recessive disease such as cystic fibrosis, cancer, etc.). The information may be able to distinguish between the following possibilities: (1) two genetic variations within the same gene on the same strand of DNA and (2) two genetic variations within the same gene but located on separate strands of DNA. Possibility (1) may indicate that one copy of the gene is normal and the individual is free of the disease, while possibility (2) may indicate that the individual has or will develop the disease, particularly if the two genetic variations are damaging to the function of the gene when present within the same gene copy. Similarly, the phasing information may also be able to distinguish between the following possibilities: (1) two genetic variations, each within a different gene on the same strand of DNA and (2) two genetic variations, each within a different gene but located on separate strands of DNA.

IX. Sequencing Polynucleotides from Small Numbers of Cells

Methods provided herein may also be used to prepare polynucleotide contained within cells in a manner that enables cell-specific information to be obtained. The methods enable detection of genetic variations (e.g., SNPs, mutations, indels, copy number variations, transversions, translocations, inversions, etc.) from very small samples, such as from samples comprising about 10-100 cells. In some cases, about 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 cells may be used in the methods described herein. In some cases, at least about 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 cells may be used in the methods described herein. In other cases, at most about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 cells may be used in the methods described herein.

In an example, a method comprises partitioning a cellular sample (or crude cell extract) such that at most one cell (or extract of one cell) is present per partition, lysing the cells, fragmenting the polynucleotides contained within the cells by any of the methods described herein, attaching the fragmented polynucleotides to barcodes, pooling, and sequencing.

As described elsewhere herein, the barcodes and other reagents may be contained within a microcapsule. These

microcapsules may be loaded into a partition (e.g., a microwell) before, after, or concurrently with the loading of the cell, such that each cell is contacted with a different microcapsule. This technique may be used to attach a unique barcode to polynucleotides obtained from each cell. The resulting tagged polynucleotides may then be pooled and sequenced, and the barcodes may be used to trace the origin of the polynucleotides. For example, polynucleotides with identical barcodes may be determined to originate from the same cell, while polynucleotides with different barcodes may be determined to originate from different cells.

The methods described herein may be used to detect the distribution of oncogenic mutations across a population of cancerous tumor cells. For example, some tumor cells may have a mutation, or amplification, of an oncogene (e.g., HER2, BRAF, EGFR, KRAS) in both alleles (homozygous), others may have a mutation in one allele (heterozygous), and still others may have no mutation (wild-type). The methods described herein may be used to detect these differences, and also to quantify the relative numbers of homozygous, heterozygous, and wild-type cells. Such information may be used, for example, to stage a particular cancer and/or to monitor the progression of the cancer and its treatment over time.

In some examples, this disclosure provides methods of identifying mutations in two different oncogenes (e.g., KRAS and EGFR). If the same cell comprises genes with both mutations, this may indicate a more aggressive form of cancer. In contrast, if the mutations are located in two different cells, this may indicate that the cancer is more benign, or less advanced.

X. Analysis of Gene Expression

Methods of the disclosure may be applicable to processing samples for the detection of changes in gene expression. A sample may comprise a cell, mRNA, or cDNA reverse transcribed from mRNA. The sample may be a pooled sample, comprising extracts from several different cells or tissues, or a sample comprising extracts from a single cell or tissue.

Cells may be placed directly into an partition (e.g., a microwell) and lysed. After lysis, the methods of the invention may be used to fragment and barcode the polynucleotides of the cell for sequencing. Polynucleotides may also be extracted from cells prior to introducing them into a partition used in a method of the invention. Reverse transcription of mRNA may be performed in a partition described herein, or outside of such a partition. Sequencing cDNA may provide an indication of the abundance of a particular transcript in a particular cell over time, or after exposure to a particular condition.

The methods presented throughout this disclosure provide several advantages over current polynucleotide processing methods. First, inter-operator variability is greatly reduced. Second, the methods may be carried out in microfluidic devices, which have a low cost and can be easily fabricated. Third, the controlled fragmentation of the target polynucleotides allows the user to produce polynucleotide fragments with a defined and appropriate length. This aids in partitioning the polynucleotides and also reduces the amount of sequence information loss due to the present of overly-large fragments. The methods and systems also provide a facile workflow that maintains the integrity of the processed polynucleotide. Additionally, the use of restriction enzymes enables the user to create DNA overhangs ("sticky ends") that may be designed for compatibility with adapters and/or barcodes.

EXAMPLES

Example 1

Generation of Non-Overlapping DNA Fragments for Sequencing

This example demonstrates a method for the generation of non-overlapping DNA fragments suitable for DNA sequencing and other downstream applications. An implementation of this method is schematically illustrated in FIG. **2**.

With reference to FIG. **2**, a target polynucleotide **101**, genomic DNA, is fragmented with the enzyme NotI, to generate a plurality of non-overlapping first polynucleotide fragments **102**. The first polynucleotide fragments are partitioned into separate microwells **103** in a microdevice such that each microwell comprises a plurality of fragments, but only a single fragment with a particular sequence **104**. The left-hand side of FIG. **2** illustrates three microwells (one is labeled **103**), each containing three exemplary unique fragments **104**, corresponding to the first polynucleotide fragments **102**. Referring again to the left-hand side of FIG. **2**, the left-most well contains fragments A1, B2, and C3, the middle well contains fragments B1, A2, and A3, and the right-most well contains fragments C1, C2, and B3.

The partitioned fragments are then further fragmented, to generate a plurality of non-overlapping second polynucleotide fragments **105**. Referring again to the left-hand side of FIG. **2**, each member of the second polynucleotide fragments is designated by its first fragment identifier (e.g., A1, B2, etc.), followed by a "−1" or a "−2". For example, first fragment A1 is fragmented to produce second fragments A1-1 and A1-2. First fragment B2 is fragmented to produce second fragments B2-1 and B2-2, and so on. For the sake of simplicity, only two second fragments are shown for each first fragment. This is, of course, not meant to be limiting, as any number of fragments may be generated at any step of the process.

The second set polynucleotide fragments are barcoded, and the barcoded sequences are pooled. Referring to the lower left-hand side of FIG. **2**, the labels [1], [2], and [3] represent three different barcode sequences used to label the second fragments **105**. The labeled sequences are designated **106**. Optionally, adapter sequences (not shown) are used to make the second fragments **105** compatible for ligation with the barcodes. The barcoding is performed while the fragments are still partitioned, before pooling. The pooled barcoded sequences are then sequenced.

With continued reference to FIG. **2**, the methods described above are then repeated, using a second rare cutter enzyme, XmaIII to digest the genomic DNA and generate a plurality of non-overlapping third polynucleotide fragments **107**. The third polynucleotide fragments and the first polynucleotide fragments are overlapping, because they are generated with different rare-cutter enzymes that cut the target polynucleotides at different sites. The third polynucleotide fragments are partitioned into separate microwells **108** in a microdevice such that each microwell comprises a plurality of fragments, but only a single fragment with a particular sequence **109**. The right-hand side of FIG. **2** illustrates three microwells (one is labeled **108**), each containing three exemplary unique fragments **109**, corresponding to the third polynucleotide fragments **107**. Referring again to the right-hand side of FIG. **2**, the left-most well contains fragments D1, E2, and F3, the middle well contains fragments E1, D2, and D3, and the right-most well contains fragments F1, F2, and E3.

With continued reference to FIG. **2**, The partitioned fragments are then further fragmented, to generate a plurality of non-overlapping fourth polynucleotide fragments **110**. The fourth polynucleotide fragments and the second polynucleotide fragments are overlapping, because they are generated by fragmenting the third and first fragments, respectively, which were generated with rare-cutter enzymes that cut the target polynucleotide at different sites, as described above. Referring again to the right-hand side of FIG. **2**, each member of the fourth set of polynucleotide fragments is designated by its third fragment identifier (e.g., D1, E2, etc.), followed by a "−1" or a "−2". For example, third fragment D1 is fragmented to produce fourth fragments D1-1 and D1-2. Third fragment E2 is fragmented to produce fourth fragments E2-1 and E2-2, and so on. For the sake of simplicity, only two fourth fragments are shown for each third fragment. This is, of course, not meant to be limiting, as any number of fragments may be generated.

The fourth polynucleotides fragments are barcoded, and the barcoded sequences are pooled. Referring to the lower right-hand side of FIG. **2**, the numbers [4], [5], and [6] represent three different barcode sequences used to label the fourth fragments **110**. The labeled sequences are designated **111**. Optionally, adapter sequences (not shown) are used to make the fourth fragments **110** compatible for ligation with the barcodes. The barcoding is performed while the fragments are still partitioned, before pooling. The pooled barcoded sequences are then sequenced.

The example above describes sequencing the barcoded second fragments separately from the barcoded fourth fragments. The barcoded second fragments and the barcoded fourth fragments may also be combined, and the combined sample may be sequenced. One or more steps of the process may be carried out in a device. The steps carried out in a device may be carried out in the same device or in different devices.

After sequencing, sequence contigs are assembled and the overlapping sequences between the second fragments and the fourth fragments are used to assemble the sequence of the genome.

Example 2

Pseudo-Random Fragmentation of Polynucleotides

A simulation was performed to evaluate the size distribution of fragments generated by a 6Mer cutter (StuI), a 4Mer cutter (CviQI), and two to seven 4Mer cutters. Random 1 Mbp DNA sequences were generated in silico and cuts were simulated based on the occurrence of the recognition sites for each of the restriction enzymes within the random sequences.

FIG. **3** shows the size distribution of a random 1 Mbp DNA sequence cut with the 6Mer cutter StuI (AGG/CCT). Fragments less than about 50 nucleotides were designated as "low yield," because they underutilize the read length capacity of sequencing instruments. Fragments less than about 200 nucleotides were designated as fragments likely to provide the most accurate data from today's sequencing technology. As described throughout this disclosure, this size range is in no way meant to be limiting, and the methods exemplified here, and described throughout this disclosure, may be used to generate fragments of any size range. Fragments from about 200 to about 400 nucleotides typically produce sequence data with systematic error for bases more than 100 bases from either fragment end. Fragments of more than about 400 nucleotides typically do not produce any useful sequence information for bases further than 200 bases from a fragment end, using today's sequencing technologies. However, this is expected to change, and the methods presented herein can be used to generate sequences of this size or larger.

As shown in FIG. **3**, 3 of 271 fragments (1.5%) were considered low yield since they were 50 bases or smaller. Fourteen fragments (5%) were considered high accuracy since they were 200 bases or smaller (i.e., each base of the fragment is within 100 bases of a restriction site and could be sequenced with high accuracy). Eleven fragments (4%) were between 200 and 400 bases and would generate data that is both accurate (0-100 bases from each end) and inaccurate (100-200 bases from each end). The remaining 246 fragments (91%) were greater than 400 bases and would generate accurate (0-100), inaccurate (100-200) and no (>200 bases from a restriction site) sequence data. Overall only 5% of the 1 Mbp random sequence was within 100 bases from a restriction site and would generate accurate sequence data.

FIG. **4** shows the results from a second simulation using the 4Mer cutter CviQI (G/TAC), instead of StuI (the 6Mer cutter described above) to simulate cutting a random 1 Mbp DNA sequence. As shown in FIG. **4**, the use of a restriction enzyme with a shorter recognition site results in more cuts, and the size distribution of the fragments is therefore shifted toward a smaller size range. In particular, as shown in FIG. **4**, 18% of fragments were considered low yield since they were 50 bases or smaller. Thirty-eight percent of fragments were considered high accuracy since they were 200 bases or smaller (i.e., each base of the fragment was within 100 bases of a restriction site and could be sequenced with high accuracy). Twenty five percent of fragments were between 200 and 400 bases and would generate data that is both accurate (0-100 bases from each end) and inaccurate (100-200 bases from each end). The remaining fragments (37%) were greater than 400 bases and would generate accurate (0-100), inaccurate (100-200) and no (>200 bases from a restriction site) sequence data. Overall 56% of the 1 Mbp random sequence was within 100 bases from a restriction site and would generate accurate sequence data. Therefore, cutting the randomly generated 1 Mbp DNA sequence with CviQI resulted in a higher percentage of fragments with nucleotides within 100 nucleotides of a restriction site than cutting with StuI (i.e., 56% vs. 5%, respectively). Cutting with CviQI is therefore expected to provide more fragments that may be fully sequenced.

Next, simulated cuts were made in a random 1 Mbp DNA sequence using combinations of one to seven different 4Mer cutters. The 4Mer cutters were: (A) CviQI (G/TAC); (B) BfaI (C/TAG); (C) HinP1I (G/CGC); (D) CviAII (C/ATG); (E) TaqαI (T/CGA); (F) MseI (T/TAA); and (G) MspI (C/CGG). The results of these simulations are shown in FIG. **5**. As shown in FIG. **5**, increasing the number of 4Mer cutter enzymes, from one to seven, increases the number of fragments with nucleotides within 100 nucleotides of a restriction site. Therefore, cutting the randomly generated 1 Mbp DNA sequence with more than one 4Mer cutter results in more fragments that may be fully sequenced than cutting with a single 4Mer cutter.

The number of enzymes used to cut a sequence can be chosen so that a particular fraction of a target nucleotide (e.g., a genomic) sequence within 100 nucleotides of a restriction enzyme is achieved. For example, the fraction of a random genome within 100 nucleotides of a restriction site for a 4Mer cutter is equal to $1-0.44^x$, where x is the number of independent 4Mer cutters. Similarly, the fraction of a random genome within 100 nucleotides of a restriction site for a 5Mer cutter is equal to $1-0.25^x$, where x is the number of independent 5Mer cutters. For a 6Mer cutter, the fraction

of a random genome within 100 nucleotides of a restriction site is equal to $1-0.95^x$, where x is the number of independent 6Mer cutters.

Table 1 shows the percentage of sequences with a length greater than 100 nucleotides for each of the seven enzymatic treatments described above. These sequences are considered those likely to result in missing data. Increasing the number of enzymes decreases the percentage of sequences greater than 100 nucleotides. The number of enzymes and their restriction site recognition length may be chosen in order to minimize the loss of sequence information from sequences greater than 100 nucleotides from a restriction site while also minimizing the generation of sequences less than 50 nucleotides, which are undesirable because the underutilize the read length capacity of sequencing instruments. The presence of these fragments may be minimized or avoided by selecting restriction enzymes that cut more rarely but at the potential price of reduced sequencing coverage of the DNA (i.e., more fragments may have bases >100 bases from a restriction site). These fragments may also be physically removed by a size selection step. Since these fragments are small and some fraction of the bases represented in the small fragments may be covered in larger fragments from other enzymes, the effect on coverage would likely be minimal.

The exemplary 4Mer cutter methods presented herein are optimized to provide fragments compatible with current DNA sequencing technology, which may achieve accurate read lengths up to about 100 nucleotides from the terminus of a fragment. One of ordinary skill in the art will readily recognize that other restriction enzymes (e.g., 5Mer cutters, 6Mer cutters, etc.) would be suitable for DNA sequencing technologies capable of accurately reading larger fragments of DNA (e.g., 300-400, or more nucleotides). The methods presented in this disclosure are, of course generalizable, and may be used to obtain DNA fragments of any size distribution compatible with present or future sequencing technology.

TABLE 1

Percentage of random 1 Mbp sequence more than 100 nucleotides from any restriction site. The letters in the first row refer to treatment with the following enzymes: (A) CviQI (G/TAC); (B) BfaI (C/TAG); (C) HinP1I (G/CGC); (D) CviAII (C/ATG); (E) TaqαI (T/CGA); (F) MseI (T/TAA); and (G) MspI (C/CGG).

A	AB	ABC	ABCD	ABCDE	ABCDEF	ABCDEFG
44.2%	20.1%	9.3%	4.2%	1.7%	0.6%	0.3%

Example 3

High Yield Adapter Ligation by Restriction Enzyme-Mediated Recycling of Undesirable Side Products

As described elsewhere herein, many downstream applications of the polynucleotide processing methods provided herein may utilize polynucleotide barcodes. An adapter may be used to provide compatible ends for the attachment of a barcode to a polynucleotide fragment (e.g., by ligation or PCR). In these cases, the desired products may be, for example:

[B]-[TPF]-[B], or

[B]-[A]-[TPF]-[A]-[B], where

[B] represents a barcode, [A] represents an adapter, and [TPF] represents a target polynucleotide fragment. However, in some cases, undesirable side products may form, for example, from the self ligation of barcodes, adapters, and/or target polynucleotide fragments. This example demonstrates one solution to this potential problem.

FIG. **6** shows a schematic of an implementation of the method described in this example. In the example shown in FIG. **6**, three polynucleotide starting materials (Genomic DNA; Adapter 1; and Adapter 2) and three enzymes (MspI; NarI; and DNA Ligase) are contained within a partition. The restriction enzyme MspI (C/CGG) recognizes the CCGG sequence occurring within the Genomic DNA sequence and cuts the Genomic DNA sequence to generate a fragment of genomic DNA. If the reaction proceeds as intended, the fragment of genomic DNA is then ligated to Adapter 1 and Adapter 2, to generate a fragment of genomic DNA flanked by ligated adapters (FIG. **6**, lower-left). This fragment with ligated adapters may then be ligated to DNA barcodes, which may also be present within the same partition (not shown).

However, the reaction described above may also result in several unwanted side products, including multimers produced by self-ligation of the fragmented genomic DNA and adapters (or other molecules, such as barcodes, which are not shown). For the sake of simplicity, FIG. **6** illustrates this concept by showing only self-ligation of fragmented genomic DNA and adapters.

One unwanted side product is a multimer of genomic DNA fragments. This may occur, for example, if genomic DNA fragments with compatible ends are ligated to each other after cutting. In FIG. **6**, cutting of Genomic DNA with MspI generates compatible ends that may be ligated by the ligase present in the partition. Similarly, Adapter 1 and Adapter 2, as shown, have compatible ligatable ends, and may also be ligated to form multimers.

As indicated in FIG. **6**, one solution to this problem is to pair one enzyme (in this example, MspI) with a second enzyme (in this example, NarI). In this example, MspI re-cuts genomic DNA multimers produced by self-ligation of genomic DNA fragments. Therefore, MspI recycles unwanted genomic DNA fragment multimers back into genomic DNA fragments, which may then be correctly ligated to the adapters. Similarly, Nan cuts multimers of Adapter 1 and Adapter 2 into monomers of Adapter 1 and monomers of Adapter 2, which may then be correctly ligated to genomic DNA fragments. This recycles unwanted adapter multimers back into the desired starting materials of Adapter 1 and Adapter 2.

The enzymes are chosen such that the desired product (i.e., the genomic DNA fragment with adapters on each end) does not contain a recognition site for either enzyme. Therefore, the product will not be re-cut by any enzyme contained within the partition. This process increases the yield of the desired product, while minimizing the number of unwanted side products and reducing the amount of starting material required to produce a desired amount of a product. As described in this disclosure, a pair of enzymes may be chosen so that one enzyme recognizes one undesirable side-product and regenerates a starting material and another recognizes another undesirable side product and regenerates another starting material, but neither enzyme recognizes the desired product. This can be done for an unlimited number of side products.

In general, one strategy for selecting such pairs is to choose two enzymes that create identical (or similar, ligatable) termini after cutting, but have recognition sequences of different lengths. FIG. **7** shows examples of such pairs of enzymes. The enzymes provided in FIG. **7**A provide sticky ends, while those provided in FIG. **7**B provide blunt ends.

The exemplary embodiment shown in FIG. **6** uses Genomic DNA and two adapters (Adapter 1 and Adapter 2) as starting materials. Therefore, in this embodiment, MspI is used not only to regenerate genomic DNA fragments after self-ligation, but also to generate the genomic DNA fragments in the first place, from Genomic DNA. Of course, this is optional, as one may introduce pre-fragmented genomic DNA into the partition and the method is still applicable.

Similarly, the embodiment shown in FIG. **6** shows two separate adapter molecules as starting materials. Adapter molecules may also be provided as a single polynucleotide sequence which is then cut by an enzyme contained within the partition (in this example, NarI) to generate ligation compatible ends for attachment to the fragmented genomic DNA. The method is also applicable to other polynucleotides described throughout this disclosure and to methods of attachment based on techniques other than ligation (e.g., attachment of an adapter or a barcode by PCR).

Pseudo-complimentary nucleotides that preferentially bind natural nucleotides over themselves (e.g., Biochemistry (1996) 35, 11170-11176; *Nucleic Acids Research* (1996) 15, 2470-2475), may also be used to minimize or avoid the formation of certain multimers, for example adapter-adapter multimers and barcode-barcode multimers. If adapters and/or barcodes (and/or other polynucleotides are synthesized using pseudo-complimentary nucleotides, they will prefer to hybridize with naturally occurring polynucleotide fragments (e.g., genomic DNA fragments) rather than themselves, therefore leading to a higher yield of the desired product.

Example 4

Provision of Reagents in Microcapsules and Directly in Microwells

As described throughout this disclosure, the polynucleotide processing methods described herein may involve the treatment of partitioned polynucleotides with a variety of reagents. These reagents may include, for example, restriction enzymes, ligases, phosphatases, kinases, barcodes, adapters, or any other reagent useful in polynucleotide processing or in a downstream application, such as sequencing. FIG. **8** shows two exemplary methods of providing reagents. On the left-hand side of FIG. **8**, reagents are provided within a microcapsule. The microcapsule that is shown in FIG. **8** has an outer shell ("3"), an intermediate non-aqueous layer ("2") and an inner aqueous drop contained within the intermediate non-aqueous layer ("1ABC+RE"). This droplet is made by a water-oil-water emulsion technique followed by polymerization of the outermost water layer ("3") to form a shell. Reagents are contained within the inner aqueous phase of the capsule. The left-hand side of FIG. **8** shows an exemplary embodiment with four reagents contained within the aqueous phase of the capsule, namely three barcode reagents (1A, 1B, and 1C), and a restriction enzyme ("RE"). The embodiment shown is merely exemplary. The reagents may be located in any part of the capsule.

The capsule is dispensed into a partition (e.g., a microwell). A target polynucleotide and a ligase are then added to the partition. The capsule is made to release its contents by exposure to a stimulus, such as a change in temperature, a solvent, or stirring. The restriction enzyme fragments the target polynucleotide and the ligase attaches the barcode reagents to the target polynucleotide fragments generated by the restriction enzyme.

The restriction digestion and ligation may proceed according to any of the methods described herein, for example by non-overlapping fragmentation techniques, by pseudo-random fragmentation methods, and/or by pairing of restriction enzymes to recycle unwanted side products into new starting products (e.g., target polynucleotide fragments and barcodes). Adapters may also be included within the microcapsule. The barcodes shown in FIG. **8** are modular. For example, barcode components 1A, 1B, and 1C may ligate to form barcode: [1A]-[1B]-[1C].

The right-hand side of FIG. **8** shows the same reagents dispensed into a microwell, followed by sealing with sealant (e.g., a wax or oil), to prevent evaporation before use. This approach may be substituted for the approach described above, where the reagents are placed within microcapsules. Both approaches are used to produce partitions (e.g., microwells) pre-loaded with reagents for DNA fragmentation and barcoding. In order to fragment and barcode DNA using reagents dispensed within a microwell, a user unseals a partition, and introduces a target polynucleotide and a ligase (or any other reagents applicable for the method the user is conducting). As described above, the restriction enzyme fragments the target polynucleotide and the ligase attaches the barcode reagents to the target polynucleotide fragments generated by the restriction enzyme. Of course, both approaches may be combined by placing certain reagents in the microwell and others in the microcapsule. While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 13

<210> SEQ ID NO 1
<211> LENGTH: 13
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

-continued

<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (5)..(9)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 1

ggccnnnnng gcc 13

<210> SEQ ID NO 2
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (4)..(8)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 2

caannnnngt gg 12

<210> SEQ ID NO 3
<211> LENGTH: 14
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (4)..(10)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 3

gaannnnnnn ttgg 14

<210> SEQ ID NO 4
<211> LENGTH: 13
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (5)..(10)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 4

gaacnnnnnn tcc 13

<210> SEQ ID NO 5
<211> LENGTH: 13
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (5)..(10)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 5

gaagnnnnnn tac 13

-continued

<210> SEQ ID NO 6
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (5)..(9)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 6

gaacnnnnnc tc 12

<210> SEQ ID NO 7
<211> LENGTH: 13
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (5)..(10)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 7

gaacnnnnnn tac 13

<210> SEQ ID NO 8
<211> LENGTH: 11
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 8

atcctttaag c 11

<210> SEQ ID NO 9
<211> LENGTH: 11
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 9

ggatcaagct a 11

<210> SEQ ID NO 10
<211> LENGTH: 11
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 10

aaattccggc a 11

<210> SEQ ID NO 11
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

-continued

<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(5)
<223> OTHER INFORMATION: a, c, t, g, unknown or other
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (10)..(19)
<223> OTHER INFORMATION: a, c, t, g, unknown or other
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (24)..(28)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 11

nnnnnccggn nnnnnnnnnc cggnnnnn 28

<210> SEQ ID NO 12
<211> LENGTH: 16
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(5)
<223> OTHER INFORMATION: a, c, t, g, unknown or other
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (12)..(16)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 12

nnnnnggcgc cnnnnn 16

<210> SEQ ID NO 13
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(5)
<223> OTHER INFORMATION: a, c, t, g, unknown or other
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (11)..(20)
<223> OTHER INFORMATION: a, c, t, g, unknown or other
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (26)..(30)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 13

nnnnnggcgg nnnnnnnnnn ccgccnnnnn 30

What is claimed is:

1. A method for nucleic acid preparation or analysis, comprising:

(a) providing:

(i) at least 1,000 gel beads;

(ii) releasably attached to each of said at least 1,000 gel beads, at least 1,000 barcode molecules comprising identical barcode sequences that are distinct from barcode sequences of at least 1,000 barcode molecules releasably attached to any other gel bead of said at least 1,000 gel beads; and

(iii) a plurality of cells each comprising a plurality of polynucleotide molecules;

(b) generating a plurality of droplets, wherein at least 1,000 droplets of said plurality of droplets each comprise:

(i) a single gel bead from said at least 1,000 gel beads; and

(ii) a single cell from said plurality of cells; and

(c) in each of said at least 1,000 droplets, using said plurality of polynucleotide molecules from said single cell and barcode molecules of said at least 1,000

barcode molecules from said single gel bead to generate a plurality of barcoded polynucleotide molecules, wherein said barcode molecules become detached from said gel bead.

2. The method of claim **1**, wherein, prior to (c), said plurality of polynucleotide molecules are released from said single cell in each of said at least 1,000 droplets.

3. The method of claim **1**, wherein said plurality of polynucleotide molecules are a plurality of messenger ribonucleic acid (mRNA) molecules.

4. The method of claim **3**, wherein (c) comprises reverse transcribing said plurality of mRNA molecules in presence of said barcode molecules to generate said plurality of barcoded polynucleotide molecules.

5. The method of claim **1**, further comprising causing said plurality of barcoded polynucleotide molecules to be released from said at least 1,000 droplets.

6. The method of claim **5**, further comprising amplifying said plurality of barcoded polynucleotide molecules by nucleic acid amplification subsequent to releasing said plurality of barcoded polynucleotide molecules from said at least 1,000 droplets.

7. The method of claim **1**, further comprising amplifying said plurality of barcoded polynucleotide molecules by nucleic acid amplification in each of said at least 1,000 droplets.

8. The method of claim **1**, further comprising causing said plurality of barcoded polynucleotide molecules or derivatives thereof to be sequenced.

9. The method of claim **1**, wherein a subset of said plurality of droplets does not include a cell.

10. The method of claim **1**, wherein a subset of said plurality of droplets does not include a gel bead.

11. The method of claim **1**, wherein in each of said at least 1,000 droplets, said barcode molecules are released from said single gel bead.

12. The method of claim **11**, wherein each of said at least 1,000 droplets further comprise a reducing agent that depolymerizes said single gel bead, thereby releasing said barcode molecules.

13. The method of claim **1**, wherein said at least 1,000 gel beads are formed by polymerization of droplets comprising said at least 1,000 barcode molecules.

14. The method of claim **1**, wherein said at least 1,000 barcode molecules are disposed within said single gel bead.

15. The method of claim **1**, wherein, in (a), said at least 1,000 gel beads are a subset of a plurality of gel beads.

16. The method of claim **15**, wherein said plurality of gel beads comprises at least 10,000 gel beads.

17. The method of claim **1**, wherein said at least 1,000 barcode molecules comprise combinatorial assemblies of sequences from sequence modules.

18. The method of claim **17**, wherein each of said combinatorial assemblies comprises a combinatorial assembly of a first sequence and a second sequence.

19. The method of claim **17**, wherein each of said combinatorial assemblies comprises a combinatorial assembly of a first sequence, a said second sequence, and a third sequence.

20. The method of claim **1**, wherein said plurality of droplets comprises at least 10,000 droplets.

21. The method of claim **1**, wherein said plurality of droplets comprises at least 100,000 droplets.

22. The method of claim **1**, wherein said at least 1,000 gel beads comprises at least 10,000 gel beads, and wherein said at least 1,000 droplets comprises at least 10,000 droplets.

23. The method of claim **1**, wherein said at least 1,000 gel beads comprises at least 100,000 gel beads, and wherein said at least 1,000 droplets comprises at least 100,000 droplets.

24. The method of claim **1**, wherein said at least 1,000 barcode molecules are at least 10,000 barcode molecules.

25. The method of claim **1**, wherein said at least 1,000 barcode molecules are at least 100,000 barcode molecules.

26. The method of claim **1**, wherein said at least 1,000 barcode molecules are at least 1,000,000 barcode molecules.

27. The method of claim **1**, wherein said plurality of polynucleotide molecules are from 10,000-100,000 polynucleotide molecules.

28. The method of claim **1**, wherein said barcode molecules become detached from said gel bead before generation of said barcoded polynucleotide molecules.

29. The method of claim **1**, wherein said barcode molecules become detached from said gel bead following generation of said barcoded polynucleotide molecules.

30. The method of claim **1**, wherein said barcode molecules become detached from said gel bead during generation of said barcoded polynucleotide molecules.

* * * * *

PROOF OF SERVICE

The undersigned hereby certifies that on August 17, 2020, I caused the foregoing ADDENDUM TO THE PRINCIPAL BRIEF FOR APPELLANT BIO-RAD (confidential and nonconfidential versions) to be electronically filed with the Clerk of the Court for the United States Court of Appeals for the Federal Circuit by using the CM/ECF system. Service will be accomplished via electronic mail. Additionally, the confidential version will be served by email on this same date.

Via CM/ECF and email

Benjamin Richards
Wayne W. Herrington
Office of the General Counsel
U.S. International Trade Commission
500 E Street, S.W.
Washington, D.C. 20436
benjamin.richards@usitc.gov
wayne.herrington@usitc.gov

Counsel for Appellee International Trade Commission

Via CM/ECF and email

Matthew D. Powers
Paul T. Ehrlich
Stefani C. Smith
Robert L. Gerrity
Jennifer K. Robinson
Utsav Gupta
Daniel M. Radke
TENSEGRITY LAW GROUP, LLP
555 Twin Dolphin Drive, Suite 650
Redwood Shores, CA 94065
Telephone: (650) 802-6000
Facsimile: (650) 802-6001
10x_tlg_service@tensegritylawgroup.com

Azra M. Hadzimehmedovic
Aaron M. Nathan
Samantha A. Jameson
TENSEGRITY LAW GROUP, LLP
8260 Greensboro Drive, Suite 260
McLean, VA 22102
Telephone: (650) 802-6000
Facsimile: (650) 802-6001
10x_tlg_service@tensegritylawgroup.com

Counsel for Appellant 10X Genomics, Inc.

Upon acceptance by the Court of the e-filed document, six confidential paper copies will be filed with the Court within the time provided in the Court's rules.

/s/ *Brian C. Cannon*

Brian C. Cannon