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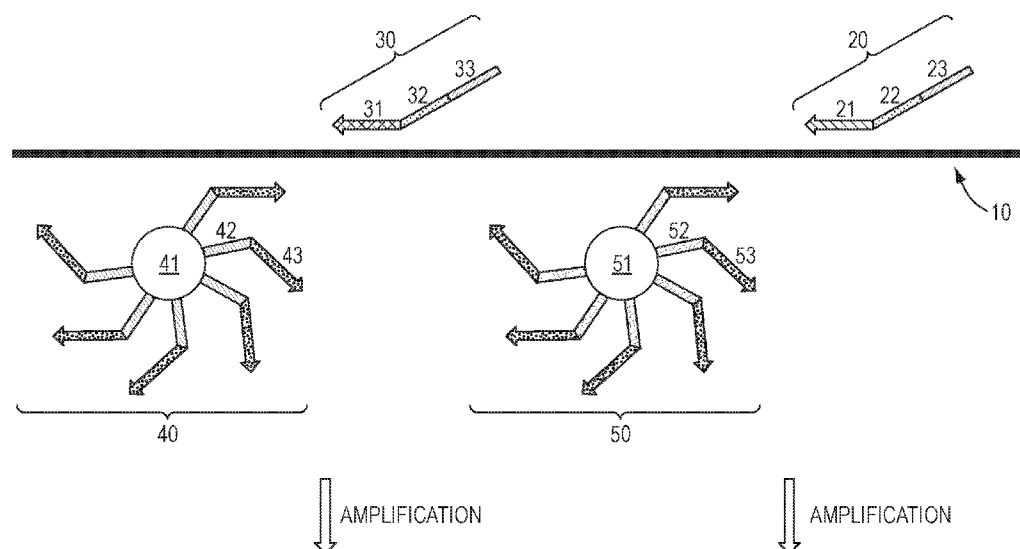


FIG. 1A

(57) Abstract: In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatuses, for conducting a stream-lined nucleic acid library preparation workflow that generally includes binding a polynucleotide (from an initial nucleic acid sample) directly to capture primers that are immobilized on a support (e.g., beads or flowcell), and amplifying the bound polynucleotide to produce a support that carries a substantially monoclonal population of the polynucleotide (or a portion thereof). The support that carries the substantially monoclonal population of the polynucleotide can be generated, using the present teachings, without performing a separate adaptor-joining step and is ready for sequencing.



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RAPID AMPLIFICATION OF NUCLEIC ACIDS

[0001] This application claims priority to U.S. Provisional Application No. 62/406,475, filed October 11, 2016, which is incorporated by reference in its entirety herein.

[0002] Throughout this application various publications, patents, patent applications, articles, books, treatises, and internet web pages, are referenced. The disclosures of the publications, patents, patent applications, articles, books, treatises, and internet web pages, in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

BACKGROUND

[0003] This disclosure relates to the field of amplification and sequencing of nucleic acids, including methods, compositions, and systems therefor. Recent advances in nucleic acid sequencing technology have reduced the cost and time necessary to obtain sequence data from a sample. Accordingly, sequence data is increasingly acquired and used in clinical and field applications, including personalized medicine and detection and identification of pathogens and other organisms. But there is still a need for greater speed in preparing a nucleic acid library for sequencing. There is also a need for a library preparation workflow that can be performed in the field (e.g., not in a laboratory), or that can be performed in a laboratory without the need for special equipment, at ambient temperature and without special equipment, where the reagents used to perform the library prep workflow do not require refrigeration or other special storage conditions.

[0004] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatuses, that can be used to solve these needs and/or provide other benefits. In some embodiments, the methods, and related compositions, systems, kits and apparatuses of the present teachings are provided in which the time from sample acquisition to data generation can be reduced. For example, such a reduction can be facilitated relative to existing library prep approaches at least by obviating a need for attachment of adaptors (e.g., universal adaptors) to polynucleotides in a sample, and/or by subjecting the sample to an amplification condition as disclosed herein.

SUMMARY

[0005] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatuses, comprising a method for amplifying nucleic acids, the method comprising: (a) forming a single reaction mixture by contacting, within a single continuous liquid phase, (i) a plurality of target polynucleotides lacking a universal adaptor sequence, (ii) a first set of beads having a plurality of a first polynucleotide-specific capture primer attached thereon, (iii) a second set of beads having a plurality of a second polynucleotide-specific capture primer attached thereon, (iv) a plurality of a first polynucleotide-specific solution-phase primer, and (v) a plurality of a second polynucleotide-specific solution phase primer; and (b) subjecting the single reaction mixture to an isothermal nucleic acid amplification condition, thereby generating from the first set of beads at least one bead attached to a first substantially monoclonal polynucleotide population containing a first portion of one of the target polynucleotides from the plurality of target polynucleotides, and generating from the second set of beads at least one bead attached to a second substantially monoclonal polynucleotide population containing a second portion of one of the target polynucleotides from the plurality of target polynucleotides.

[0006] In some embodiments, the plurality of target polynucleotides is isolated from a biological fluid, cell culture, or solid tissue.

[0007] In some embodiments, the plurality of target polynucleotides is isolated from a biological fluid and the biological fluid comprises blood, serum, plasma, saliva, sputum, sweat, tears, lavage fluid, amniotic fluid, cerebrospinal fluid, ascites, urine, or semen.

[0008] In some embodiments, the plurality of target polynucleotides lacking an adaptor sequence (e.g., universal adaptor sequence) comprises genomic DNA. For example, the genomic DNA can be un-fragmented or fragmented, but the genomic DNA is not joined to an adaptor sequence. In some embodiments, the plurality of target polynucleotides are attached at one or both ends to at least one adaptor sequence (e.g., universal adaptor sequence). In some embodiments, the plurality of polynucleotides is isolated from any organism including human, canine, feline, bovine, equine, murine, porcine, caprine, lupine, ranine, piscine, simian, ape, plant, insect, bacteria, virus or fungus. In some embodiments, the plurality of polynucleotides can originate from water, soil or food.

[0009] In some embodiments, the first polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a first target polynucleotide of the plurality of target polynucleotides, and wherein the second polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a second target polynucleotide of the plurality of target polynucleotides. In some embodiments, the first and second polynucleotide-specific capture primers hybridize to different locations/regions of the same polynucleotide molecule which contains the first and second target polynucleotide sequences.

[0010] In some embodiments, the first polynucleotide-specific capture primer further comprises at least one universal sequence that is capable of hybridizing with an amplification primer and/or sequencing primer, and wherein the second polynucleotide-specific capture primer further comprises at least one universal sequence that is capable of hybridizing with an amplification primer and/or sequencing primer.

[0011] In some embodiments, the first polynucleotide-specific capture primer comprises at least one unique identifier sequence, and wherein the second polynucleotide-specific capture primer comprises at least one unique identifier sequence.

[0012] In some embodiments, the plurality of target polynucleotides contains at least a first target polynucleotide and a second target polynucleotide having different sequences.

[0013] In some embodiments, the first polynucleotide-specific solution-phase primer is capable of hybridizing with at least a portion of the plurality of target polynucleotides. In some embodiments, the second polynucleotide-specific solution-phase primer is capable of hybridizing with at least a portion of the plurality of target polynucleotides.

[0014] In some embodiments, the first polynucleotide-specific solution-phase primer comprises at least one universal sequence which is capable of hybridizing with an amplification primer and/or sequencing primer and the first polynucleotide-specific solution-phase primer optionally includes at least one unique identifier sequence, and wherein the second polynucleotide-specific solution-phase primer comprises at least one universal sequence which is capable of hybridizing with an amplification primer and/or sequencing primer and the second polynucleotide-specific solution-phase primer optionally includes at least one unique identifier sequence.

[0015] In some embodiments, the first polynucleotide-specific capture primer and the first polynucleotide-specific solution-phase primer hybridize to different portions of the same target polynucleotide from the plurality of target polynucleotides.

[0016] In some embodiments, the portion of the target polynucleotide to which the first polynucleotide-specific capture primer hybridizes is located on a first strand of the target polynucleotide in its double-stranded form, and the portion of the target polynucleotide to which the first polynucleotide-specific solution-phase primer hybridizes is located on a second strand of the target polynucleotide in its double-stranded form.

[0017] In some embodiments, the second polynucleotide-specific capture primer and the second polynucleotide-specific solution-phase primer hybridize to different portions of the same target polynucleotide.

[0018] In some embodiments, the portion of the target polynucleotide to which the second polynucleotide-specific capture primer hybridizes is located on a first strand of the target polynucleotide in its double-stranded form, and the portion of the target polynucleotide to which the second polynucleotide-specific solution-phase primer hybridizes is located on a second strand of the target polynucleotide in its double-stranded form.

[0019] In some embodiments, the single reaction mixture comprises a polymerase and a plurality of nucleotides.

[0020] In some embodiments, the single reaction mixture comprises a recombinase. In some embodiments, the recombinase comprises uvsX. In some embodiments, the single reaction mixture comprises at least one recombinase loading protein and/or at least one single-stranded binding protein. In some embodiments, the at least one recombinase loading protein comprises uvsY. In some embodiments, the at least one single-stranded binding protein comprises gp32.

[0021] In some embodiments, the method further comprises: sequencing the first substantially monoclonal polynucleotide population and the second substantially monoclonal polynucleotide population.

[0022] In some embodiments, the method further comprises: depositing onto a first reaction chamber of a support the at least one bead which is attached to the first substantially monoclonal polynucleotide population, and depositing onto a second reaction chamber of the same support the at least one bead which is attached to the second substantially monoclonal polynucleotide

population, wherein the support contains an array of reaction chambers which includes the first and second reaction chambers.

[0023] In some embodiments, the individual reaction chambers in the array, including the first and second reaction chambers, are operatively coupled to at least one sensor that detects a change in the level or abundance of hydrogen ions, protons, or phosphate groups.

[0024] In some embodiments, the method further comprises: sequencing the first substantially monoclonal polynucleotide population at the first reaction chamber, and sequencing the second substantially monoclonal polynucleotide population at the second reaction chamber.

[0025] In some embodiments, the sequencing comprises: detecting a nucleotide incorporation byproduct using the at least one sensor which is operatively coupled to the first reaction chamber, and wherein sequencing comprises detecting a nucleotide incorporation byproduct using the at least one sensor which is operatively coupled to the second reaction chamber.

[0026] In some embodiments, a first set of beads which includes at least one bead attached to a first substantially monoclonal polynucleotide population containing a first portion of one of the target polynucleotides, and a second set of beads which includes at least one bead attached to a second substantially monoclonal polynucleotide population containing a second portion of one of the target polynucleotides, wherein the first and second set of beads are prepared by according any of the methods of the present teachings.

[0027] In some embodiments, a composition, comprises: (i) a plurality of target polynucleotides lacking a universal adaptor sequence, (ii) a plurality of different sets of beads, wherein each set of beads has a plurality of a polynucleotide-specific capture primer attached thereon, wherein the polynucleotide-specific capture primer differs in sequence between at least two sets of beads, and wherein at least one of the polynucleotide-specific capture primers hybridizes with at least a portion of at least one of the target polynucleotides, (iii) a plurality of polynucleotide-specific solution phase primers, (iv) a polymerase, (v) a plurality of nucleotides, (vi) optionally a uvsX recombinase, (vii) optionally a uvsY recombinase loading protein, and (viii) optionally a gp32 single stranded binding protein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] **Figure 1A** is a non-limiting example of methods for amplifying nucleic acids by contacting (i) a target polynucleotide (**10**), (ii) a plurality of supports having a plurality of a polynucleotide-specific capture primer attached thereon (**40**) and (**50**), and (iii) a plurality of polynucleotide-specific solution-phase primers (**20**) and (**30**), under amplification conditions.

[0029] **Figure 1B** is a non-limiting example of a set of supports attached to a substantially monoclonal polynucleotide population containing a portion of the target polynucleotide (**60**) and (**70**).

[0030] **Figure 2** is a non-limiting example of a procedure that can be used to generate beads that are attached to a plurality of polynucleotide-specific capture primers.

DESCRIPTION

[0031] This description and exemplary embodiments should not be taken as limiting. For the purposes of this specification and appended claims, unless otherwise indicated, all numbers expressing quantities, percentages, or proportions, and other numerical values used in the specification and claims, are to be understood as being modified in all instances by the term “about,” to the extent they are not already so modified. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

[0032] It is noted that, as used in this specification and the appended claims, the singular forms “a,” “an,” and “the,” and any singular use of any word, include plural referents unless expressly and unequivocally limited to one referent. As used herein, the term “include” and its grammatical variants are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that can be substituted or added to the listed items.

[0033] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatuses, for conducting a stream-lined nucleic acid library preparation workflow that generally includes combining surface-templating and adaptor-joining reactions. In some embodiments, the stream-lined nucleic acid library preparation workflow

comprises binding a polynucleotide (from an initial nucleic acid sample) directly to capture primers that are immobilized on a support (e.g., beads, particles or flowcell), and amplifying the bound polynucleotide to produce a support that carries a substantially monoclonal population of the polynucleotide (or a portion thereof). In some embodiments, the amplifying step can be performed under isothermal conditions. The support that carries the substantially monoclonal population of the polynucleotide can be generated, using the present teachings, without performing a separate adaptor-joining step and is ready for sequencing. The support that carries the substantially monoclonal population of the polynucleotide can be subjected to any type of massively parallel sequencing procedure to obtain sequencing data, for example of specific loci, or loci comprising polymorphisms such as single nucleotide polymorphisms or signature nucleotides that identify a particular strain, species, or other taxon, within complex samples such as genomic DNA and total RNA. The sample that contains the initial nucleic acid can be a biological sample. In some embodiments, the support comprises a substantially planar support, a flowcell, a plurality of wells, a particle or a bead. The terms “bead” and “particle” may be used interchangeably throughout the present teachings.

[0034] The methods provided in the present teachings offer several advantages over existing library preparation and support templating workflows, because the methods can be performed in the field or the laboratory, without the need for special equipment, at ambient temperature. In some embodiments, the methods, and related compositions, systems, kits and apparatuses, can be used to detect and identify pathogens of infectious diseases, sepsis, or food contamination, or for human identification, or for haplotyping, or for determining phased or un-phased genotypes for haplotype analysis, or for generating overlapping sequencing reads for long reads assembly.

[0035] In some embodiments, the methods, and related compositions, systems, kits and apparatuses, for conducting a stream-lined nucleic acid library preparation workflow can be practiced in a multiplex manner with multiple sets of supports, where the supports between each set are immobilized with different capture primers. For example, the multiple sets of supports includes at least a first set of supports which carries a plurality of immobilized first capture primers, and a second set of supports which carries a plurality of immobilized second capture primers, and so on with multiple different sets of supports, and wherein the first and second capture primers differ in sequence from each other and from the other capture primers that are immobilized to the different sets of supports.

[0036] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids comprising: forming a single reaction mixture by contacting, within a single continuous liquid phase, (i) a biological fluid containing a plurality of target polynucleotides, (ii) a first set of particles having a plurality of a first polynucleotide-specific capture primer attached thereon, and (iii) a plurality of a first polynucleotide-specific solution-phase primer. In some embodiments, the methods further comprise subjecting the single reaction mixture to a nucleic acid amplification condition, thereby generating from the first set of particles at least one particle attached to a substantially monoclonal polynucleotide population containing a portion of one of the target polynucleotides from the plurality of target polynucleotides. In some embodiments, the biological fluid comprises blood, serum, plasma, saliva, sputum, sweat, tears, lavage fluid, amniotic fluid, cerebrospinal fluid, ascites, urine, or semen. In some embodiments, the plurality of target polynucleotides comprises chromatin, genomic DNA, RNA, fragmented DNA, fragmented RNA, amplified DNA, amplified RNA, or nucleic acid molecules from an RNA or DNA library (where the library molecules optionally include at least one adaptor sequence). Optionally, the plurality of target polynucleotides contains an adaptor sequence (e.g., universal adaptor sequence) or lack an adaptor sequence.

[0037] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids comprising: forming a single reaction mixture by contacting, within a single continuous liquid phase, (i) a plurality of target polynucleotides, (ii) a first set of particles having a plurality of a first polynucleotide-specific capture primer attached thereon, and (iii) a plurality of a first polynucleotide-specific solution-phase primer. In some embodiments, the methods further comprise subjecting the single reaction mixture to a nucleic acid amplification condition, thereby generating from the first set of particles at least one particle attached to a substantially monoclonal polynucleotide population containing a portion of one of the target polynucleotides from the plurality of target polynucleotides. In some embodiments, the plurality of target polynucleotides contains an adaptor sequence (e.g., universal adaptor sequence) or lack an adaptor sequence. In some embodiments, the plurality of target polynucleotides is isolated from a biological fluid, cell culture, or solid tissue. In some embodiments, the plurality of target polynucleotides is isolated

from a biological fluid and the biological fluid comprises blood, serum, plasma, saliva, sputum, sweat, tears, lavage fluid, amniotic fluid, cerebrospinal fluid, ascites, urine, or semen.

[0038] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids comprising: (a) depositing a plurality of particles onto a support having an array of reaction sites, where individual reaction site receives one or more particles. In some embodiments, individual particles in the plurality of particles include a plurality of polynucleotide-specific capture primers attached thereon. In some embodiments, the methods further comprise (b) contacting the deposited particles with (i) a plurality of target polynucleotides and (ii) a plurality of a polynucleotide-specific solution-phase primers. In some embodiments, the methods further comprise (c) subjecting the deposited particles to a nucleic acid amplification condition, thereby generating from the deposited particles at least one particle attached to a substantially monoclonal polynucleotide population containing a portion of one of the plurality of target polynucleotides. In some embodiments, individual target polynucleotides among the plurality of a target polynucleotide contain an adaptor sequence (e.g., universal adaptor sequence) or lack an adaptor sequence. In some embodiments, the plurality of target polynucleotides is isolated from a biological fluid, cell culture, or solid tissue. In some embodiments, the plurality of target polynucleotides is isolated from a biological fluid and the biological fluid comprises blood, serum, plasma, saliva, sputum, sweat, tears, lavage fluid, amniotic fluid, cerebrospinal fluid, ascites, urine, or semen. In some embodiments, the particles are deposited onto a support having at least one reaction site or an array of reaction sites. In some embodiments, the amplification reaction is conducted at the one reaction site, or at multiple reaction sites within the array. Optionally, the one reaction site, or at least one reaction site in the array, is operatively coupled to one or more sensors. Optionally, the sensor can detected at least one nucleotide incorporation byproduct, including hydrogen ions, hydroxyl ions, pyrophosphate, charge transfer, or heat. Optionally, the sensor detects a change in pH. Optionally, the sensor comprises a field effect transistor (FET). Optionally, the sensor comprises a chemical field effect transistor (chemFET). Optionally, the sensor comprises an ion-sensitive field effect transistor (ISFET).

[0039] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include a first set of particles which is a set of microparticles or a set of beads. In some embodiments, the first set of particles has a plurality of

a first polynucleotide-specific capture primers attached thereon, where the first polynucleotide-specific capture primers are optionally covalently attached thereon. In some embodiments, the polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a target polynucleotide of the plurality of target polynucleotides. In some embodiments, a 3' region of the polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a target polynucleotide of the plurality of target polynucleotides.

[0040] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include a first polynucleotide-specific capture primer which comprises at least one universal sequence. In some embodiments, at least one universal sequence is capable of hybridizing with an amplification primer and/or sequencing primer. In some embodiments, the universal sequence does not substantially hybridize with a target polynucleotide.

[0041] In some embodiments, the first polynucleotide-specific capture primer comprises at least one unique identifier sequence. In some embodiments, the unique identifier sequence is a barcode sequence which is a sample-specific barcode sequence.

[0042] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include a first polynucleotide-specific solution-phase primer which is capable of hybridizing with at least a portion of a target polynucleotide of the plurality of target polynucleotides. In some embodiments, a 3' region of the first polynucleotide-specific solution-phase primer is capable of hybridizing with at least a portion of a target polynucleotide of the plurality of target polynucleotides. In some embodiments, the first polynucleotide-specific solution-phase primer comprises at least one universal sequence. In some embodiments, at least one universal sequence is capable of hybridizing with an amplification primer and/or sequencing primer. In some embodiments, the universal sequence does not substantially hybridize with a target polynucleotide. In some embodiments, the first polynucleotide-specific solution-phase primer comprises at least one unique identifier sequence. In some embodiments, the unique identifier sequence is a sample-specific barcode sequence. In some embodiments, the first polynucleotide-specific solution-phase primer is a reverse amplification primer. In some embodiments, the first polynucleotide-specific solution-phase primer is a tailed primer.

[0043] In some embodiments, the first polynucleotide-specific capture primer and the first polynucleotide-specific solution-phase primer hybridize to different portions of the same target polynucleotide. In some embodiments, the portion of the target polynucleotide to which the first polynucleotide-specific capture primer hybridizes and the portion of the target polynucleotide to which the first polynucleotide-specific solution-phase primer hybridizes are separated by 10 to 2000 base pairs, or 10 to 1500 base pairs, or 10 to 1000 base pairs, or 10 to 500 base pairs, or 50 to 500 base pairs on the target polynucleotide in its double-stranded form. In some embodiments, the portion of the target polynucleotide to which the first polynucleotide-specific capture primer hybridizes is on a first strand of the target polynucleotide in its double-stranded form, and the portion of the target polynucleotide to which the first polynucleotide-specific solution-phase primer hybridizes is on a second strand of the target polynucleotide in its double-stranded form.

[0044] In some embodiments, the nucleic acid amplification condition comprises one primer extension reaction or multiple primer extension reactions. In some embodiments, the nucleic acid amplification condition comprises an isothermal amplification condition or a thermocycling amplification condition.

[0045] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include a single reaction mixture comprising a polymerase and a plurality of nucleotides. In some embodiments, the single reaction mixture comprises a recombinase. In some embodiments, the recombinase is uvsX. In some embodiments, the single reaction mixture comprises at least one recombinase accessory protein. In some embodiments, the single reaction mixture comprises at least one recombinase loading protein and/or at least one single-stranded binding protein. In some embodiments, at least one recombinase loading protein is uvsY. In some embodiments, at least one single-stranded binding protein is gp32.

[0046] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids further include sequencing the substantially monoclonal polynucleotide population. In some embodiments, the sequencing comprises contacting a sequencing primer to the substantially monoclonal polynucleotide population. In some embodiments, the sequencing primer is capable of hybridizing to a portion of a polynucleotide of the substantially monoclonal polynucleotide population. In some embodiments, at least one

particle attached to a substantially monoclonal polynucleotide population is deposited on a support having at least one reaction site operatively coupled to a sensor. In some embodiments, at least one particle attached to a substantially monoclonal polynucleotide population is deposited on a reaction site of a support having an array of reaction sites. In some embodiments, the individual reaction sites in the array are operatively coupled to at least one sensor. In some embodiments, the method comprises sequencing the substantially monoclonal polynucleotide population, and the sequencing is conducted at the reaction site. In some embodiments, the sequencing comprises detecting at least one nucleotide incorporation byproduct using the at least one sensor operatively coupled to the reaction site. In some embodiments, at least one nucleotide incorporation byproduct is selected from hydrogen ions, hydroxyl ions, pyrophosphate, charge transfer, and heat. In some embodiments, the sensor detects a change in pH. In some embodiments, the sensor comprises a field effect transistor (FET). In some embodiments, the sensor comprises a chemical field effect transistor (chemFET). In some embodiments, the sensor comprises an ion-sensitive field effect transistor (ISFET).

[0047] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids, comprise: (a) forming a single reaction mixture by contacting, within a single continuous liquid phase, (i) a biological fluid containing a plurality of target polynucleotides, (ii) a first set of particles having a plurality of a first polynucleotide-specific capture primer attached thereon, (iii) a second set of particles having a plurality of a second polynucleotide-specific capture primer attached thereon, (iv) a plurality of a first polynucleotide-specific solution-phase primer, and (v) a plurality of a second polynucleotide-specific solution phase primer. In some embodiments, the methods further comprise: (b) subjecting the single reaction mixture to a nucleic acid amplification condition. In some embodiments, the nucleic acid amplification condition generates, from the first set of particles, at least one particle attached to a first substantially monoclonal polynucleotide population containing a first portion of one of the target polynucleotides from the plurality of target polynucleotides. In some embodiments, the nucleic acid amplification condition, generates from the second set of particles, at least one particle attached to a second substantially monoclonal polynucleotide population containing a second portion of one of the target polynucleotides from the plurality of target polynucleotides. In some embodiments, the target polynucleotides lack an adaptor sequence.

[0048] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids, comprise: (a) forming a single reaction mixture by contacting, within a single continuous liquid phase, (i) a plurality of target polynucleotides, (ii) a first set of particles having a plurality of a first polynucleotide-specific capture primer attached thereon, (iii) a second set of particles having a plurality of a second polynucleotide-specific capture primer attached thereon, (iv) a plurality of a first polynucleotide-specific solution-phase primer, and (v) a plurality of a second polynucleotide-specific solution phase primer. In some embodiments, the methods further comprise: (b) subjecting the single reaction mixture to a nucleic acid amplification condition. In some embodiments, the nucleic acid amplification condition generates, from the first set of particles, at least one particle attached to a first substantially monoclonal polynucleotide population containing a first portion of one of the target polynucleotides from the plurality of target polynucleotides. In some embodiments, the nucleic acid amplification condition generates, from the second set of particles, at least one particle attached to a second substantially monoclonal polynucleotide population containing a second portion of one of the target polynucleotides from the plurality of target polynucleotides. In some embodiments, the plurality of a target polynucleotide contains an adaptor sequence (e.g., universal adaptor sequence) or lack an adaptor sequence.

[0049] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids, comprise: (a) depositing onto a support having an array of reaction sites, including a first and second reaction site, (i) a first particle having a plurality of a first polynucleotide-specific capture primer attached thereon, where the first particle is deposited onto a first reaction site, (ii) a second particle having a plurality of a second polynucleotide-specific capture primer attached thereon, where the second particle is deposited onto a second reaction site. In some embodiments, the methods further comprise (b) contacting the deposited first and second particles with (i) a plurality of target polynucleotides, including a first and a second target polynucleotide, and (ii) a plurality of a first polynucleotide-specific solution-phase primers, and (ii) a plurality of a second polynucleotide-specific solution phase primers. In some embodiments, the methods further comprise (c) subjecting the deposited particles to a nucleic acid amplification condition, thereby (i) generating from the first particle, a first particle attached to a first substantially monoclonal polynucleotide population containing a first portion of the first target polynucleotide, and (ii) generating from the second particle, a

second particle attached to a second substantially monoclonal polynucleotide population containing a first portion of the second target polynucleotide. In some embodiments, the plurality of target polynucleotides is isolated from a biological fluid, cell culture, or solid tissue. In some embodiments, the plurality of a target polynucleotide contains an adaptor sequence (e.g., universal adaptor sequence) or lack an adaptor sequence. In some embodiments, the plurality of target polynucleotides is isolated from a biological fluid and the biological fluid comprises blood, serum, plasma, saliva, sputum, sweat, tears, lavage fluid, amniotic fluid, cerebrospinal fluid, ascites, urine, or semen. In some embodiments, the particles are deposited onto a support having at least one reaction site or an array of reaction sites. In some embodiments, the amplification reaction is conducted at the reaction site. Optionally, the one reaction site, or at least one reaction site in the array, is operatively coupled to one or more sensors. Optionally, the sensor can detect at least one nucleotide incorporation byproduct, including hydrogen ions, hydroxyl ions, pyrophosphate, charge transfer, or heat. Optionally, the sensor detects a change in pH. Optionally, the sensor comprises a field effect transistor (FET). Optionally, the sensor comprises a chemical field effect transistor (chemFET). Optionally, the sensor comprises an ion-sensitive field effect transistor (ISFET).

[0050] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include a plurality of target polynucleotides which is isolated from a biological fluid, cell culture, solid tissue or the plurality of target polynucleotides are isolated from a single cell. In some embodiments, the plurality of target polynucleotides is isolated from a biological fluid and the biological fluid comprises blood, serum, plasma, saliva, sputum, sweat, tears, lavage fluid, amniotic fluid, cerebrospinal fluid, ascites, urine, or semen.

[0051] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include a first set of particles which is a set of microparticles or a set of beads. In some embodiments, a second set of particles is a set of microparticles or a set of beads. In some embodiments, the first set of particles is a set of microparticles or a set of beads, and the second set of particles is a set of microparticles or a set of beads. In some embodiments, the first set of particles has a plurality of a first polynucleotide-specific capture primers attached thereon and the second set of particles has a plurality of a second polynucleotide-specific capture primers attached thereon. In some embodiments, the first polynucleotide-specific capture primers are optionally covalently attached to the first set of

particles. In some embodiments, the second polynucleotide-specific capture primers are optionally covalently attached to the second set of particles.

[0052] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include a first polynucleotide-specific capture primer which is capable of hybridizing with at least a portion of a first target polynucleotide of the plurality of target polynucleotides. In some embodiments, a 3' region of the first polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a first target polynucleotide of the plurality of target polynucleotides. In some embodiments, the first polynucleotide-specific capture primer comprises at least one universal sequence. In some embodiments, at least one universal sequence is capable of hybridizing with an amplification primer and/or sequencing primer. In some embodiments, at least one universal sequence is capable of hybridizing with an amplification primer and/or sequencing primer. In some embodiments, the universal sequence does not substantially hybridize with a target polynucleotide. In some embodiments, the first polynucleotide-specific capture primer comprises at least one unique identifier sequence. In some embodiments, the unique identifier sequence is a sample-specific barcode sequence.

[0053] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include a second polynucleotide-specific capture primer which is capable of hybridizing with at least a portion of a second target polynucleotide of the plurality of target polynucleotides, or is capable of hybridizing with at least a portion of the same target polynucleotide (of the plurality of target polynucleotides) to which the first polynucleotide-specific capture primer hybridizes to. In some embodiments, a 3' region of the second polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a second target polynucleotide of the plurality of target polynucleotides, or is capable of hybridizing with at least a portion of the same target polynucleotide (of the plurality of target polynucleotides) to which the first polynucleotide-specific capture primer hybridizes to. In some embodiments, the second polynucleotide-specific capture primer comprises at least one universal sequence. In some embodiments, at least one universal sequence is capable of hybridizing with an amplification primer and/or sequencing primer. In some embodiments, at least one universal sequence is capable of hybridizing with an amplification primer and/or sequencing primer. In some embodiments, the universal sequence does not substantially hybridize with a target

polynucleotide. In some embodiments, the second polynucleotide-specific capture primer comprises at least one unique identifier sequence. In some embodiments, the unique identifier sequence is a sample-specific barcode sequence.

[0054] In some embodiments, the first target polynucleotide and the second target polynucleotide are different, for example they have different sequences and/or lengths. In some embodiments, the first and second target polynucleotide sequences are located on the same nucleic acid molecule, or are located on different nucleic acid molecules.

[0055] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include a first polynucleotide-specific solution-phase primer which is capable of hybridizing with at least a portion of a first target polynucleotide of the plurality of target polynucleotides. In some embodiments, a 3' region of the first polynucleotide-specific solution-phase primer is capable of hybridizing with at least a portion of a first target polynucleotide of the plurality of target polynucleotides. In some embodiments, the first polynucleotide-specific solution-phase primer comprises at least one universal sequence. In some embodiments, at least one universal sequence is capable of hybridizing with an amplification primer and/or sequencing primer. In some embodiments, the universal sequence does not substantially hybridize with a target polynucleotide. In some embodiments, the first polynucleotide-specific solution-phase primer comprises at least one unique identifier sequence. In some embodiments, the unique identifier sequence is a sample-specific barcode sequence. In some embodiments, the first polynucleotide-specific solution-phase primer is a reverse amplification primer. In some embodiments, the first polynucleotide-specific solution-phase primer is a tailed primer.

[0056] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include a second polynucleotide-specific solution-phase primer which is capable of hybridizing with at least a portion of a second target polynucleotide of the plurality of target polynucleotides, or is capable of hybridizing with at least a portion of the same target polynucleotide (of the plurality of target polynucleotides) to which the first polynucleotide-specific solution-phase primer hybridizes to. In some embodiments, a 3' region of the second polynucleotide-specific solution-phase primer is capable of hybridizing with at least a portion of a second target polynucleotide of the plurality of target polynucleotides, or is capable of hybridizing with at least a portion of the same target polynucleotide (of the plurality

of target polynucleotides) to which the first polynucleotide-specific solution-phase primer hybridizes to. In some embodiments, the second polynucleotide-specific solution-phase primer comprises at least one universal sequence. In some embodiments, at least one universal sequence is capable of hybridizing with an amplification primer and/or sequencing primer. In some embodiments, the universal sequence does not substantially hybridize with a target polynucleotide. In some embodiments, the second polynucleotide-specific solution-phase primer comprises at least one unique identifier sequence. In some embodiments, the unique identifier sequence is a sample-specific barcode sequence. In some embodiments, the second polynucleotide-specific solution-phase primer is a reverse amplification primer. In some embodiments, the second polynucleotide-specific solution-phase primer is a tailed primer.

[0057] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids comprise hybridizing the first polynucleotide-specific capture primer and the first polynucleotide-specific solution-phase primer to different portions of the same target polynucleotide (e.g., a first target polynucleotide). In some embodiments, the portion of the target polynucleotide to which the first polynucleotide-specific capture primer hybridizes and the portion of the target polynucleotide to which the first polynucleotide-specific solution-phase primer hybridizes are separated by 10 to 2000 base pairs, or 10 to 1500 base pairs, or 10 to 1000 base pairs, or 10 to 500 base pairs, or 50 to 500 base pairs on the target polynucleotide in its double-stranded form. In some embodiments, the portion of the target polynucleotide to which the first polynucleotide-specific capture primer hybridizes is on a first strand of the target polynucleotide in its double-stranded form, and the portion of the target polynucleotide to which the first polynucleotide-specific solution-phase primer hybridizes is on a second strand of the target polynucleotide in its double-stranded form.

[0058] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids comprise hybridizing the second polynucleotide-specific capture primer and the second polynucleotide-specific solution-phase primer to different portions of the same target polynucleotide (e.g., a second target polynucleotide), where the first and second target polynucleotides are the same polynucleotide molecule or different polynucleotide molecules. In some embodiments, the portion of the target polynucleotide to which the second polynucleotide-specific capture primer hybridizes and the portion of the target polynucleotide to which the second polynucleotide-specific solution-phase primer hybridizes are separated by 10

to 2000 base pairs, or 10 to 1500 base pairs, or 10 to 1000 base pairs, or 10 to 500 base pairs, or 50 to 500 base pairs on the target polynucleotide in its double-stranded form. In some embodiments, the portion of the target polynucleotide to which the second polynucleotide-specific capture primer hybridizes is on a first strand of the target polynucleotide in its double-stranded form, and the portion of the target polynucleotide to which the second polynucleotide-specific solution-phase primer hybridizes is on a second strand of the target polynucleotide in its double-stranded form.

[0059] In some embodiments, the nucleic acid amplification condition comprises a primer extension reaction or multiple primer extension reactions. In some embodiments, the nucleic acid amplification condition comprises an isothermal amplification condition or a thermocycling amplification condition. In some embodiments, the single reaction mixture comprises a polymerase and a plurality of nucleotides.

[0060] In some embodiments, the single reaction mixture comprises a recombinase. In some embodiments, the recombinase is uvsX. In some embodiments, the single reaction mixture comprises at least one recombinase accessory protein. In some embodiments, the single reaction mixture comprises at least one recombinase loading protein and/or at least one single-stranded binding protein. In some embodiments, at least one recombinase loading protein is uvsY. In some embodiments, at least one single-stranded binding protein is gp32.

[0061] In some embodiments, the single reaction mixture further comprises: an inorganic phosphatase (e.g., from yeast), PEG and/or trehalose.

[0062] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids further comprise sequencing at least one of the substantially monoclonal polynucleotide populations. In some embodiments, the method comprises sequencing the first substantially monoclonal polynucleotide population and the second substantially monoclonal polynucleotide population. In some embodiments, the sequencing comprises contacting at least one sequencing primer to at least one substantially monoclonal polynucleotide population. In some embodiments, at least one sequencing primer is capable of hybridizing to a portion of a polynucleotide of the first and/or second substantially monoclonal polynucleotide population. In some embodiments, the method comprises contacting a universal sequencing primer to the first substantially monoclonal polynucleotide population

and the second substantially monoclonal polynucleotide population. In some embodiments, the universal sequencing primer does not substantially hybridize to a target polynucleotide.

[0063] In some embodiments, at least one particle attached to a first substantially monoclonal polynucleotide population is deposited on a support having at least one reaction site operatively coupled to a sensor. In some embodiments, at least one particle attached to a second substantially monoclonal polynucleotide population is deposited on a support having at least one reaction site operatively coupled to a sensor. In some embodiments, at least one particle attached to a first substantially monoclonal polynucleotide population is deposited on a first reaction site of a support having an array of reaction sites. In some embodiments, at least one particle attached to a second substantially monoclonal polynucleotide population is deposited on a second reaction site of a support having an array of reaction sites. In some embodiments, at least one particle attached to a first substantially monoclonal polynucleotide population is deposited on a first reaction site and at least one particle attached to a second substantially monoclonal polynucleotide population is deposited on a second reaction site, where the first and second reaction sites are located on the same array of reaction sites. In some embodiments, the individual reaction sites in the array are operatively coupled to at least one sensor. In some embodiments, each individual reaction site in the array is operatively coupled to a different sensor. In some embodiments, the method comprises sequencing at least one substantially monoclonal polynucleotide population, and wherein the sequencing is conducted at the reaction site. In some embodiments, the method comprises sequencing the first substantially monoclonal polynucleotide population and the second substantially monoclonal polynucleotide population. In some embodiments, the sequencing comprises detecting a nucleotide incorporation byproduct using the at least one sensor operatively coupled to the reaction site. In some embodiments, the nucleotide incorporation byproduct is selected from hydrogen ions, hydroxyl ions, pyrophosphate, charge transfer, and heat. In some embodiments, the sensor detects a change in pH. In some embodiments, the sensor comprises a field effect transistor (FET). In some embodiments, the sensor comprises a chemical field effect transistor (chemFET). In some embodiments, the sensor comprises an ion-sensitive field effect transistor (ISFET).

[0064] In some embodiments, the first substantially monoclonal polynucleotide population and the second substantially monoclonal polynucleotide population differ in sequence.

[0065] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids comprise: (a) forming a single reaction mixture by contacting, within a single continuous liquid phase, (i) a biological fluid containing a plurality of target polynucleotides, (ii) a plurality of different sets of particles, wherein each set of particles has a plurality of a polynucleotide-specific capture primer attached thereon, wherein the polynucleotide-specific capture primer differs in sequence between at least two sets of particles, and (iii) a plurality of different polynucleotide-specific solution-phase primers. In some embodiments, the methods further comprise: (b) subjecting the single reaction mixture to a nucleic acid amplification condition, thereby generating from a least one set of particles at least one particle attached to a substantially monoclonal polynucleotide population containing a portion of one of the target polynucleotides from the plurality of target polynucleotides. In some embodiments, the plurality of a target polynucleotide contains an adaptor sequence (e.g., universal adaptor sequence) or lack an adaptor sequence.

[0066] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids comprise: (a) forming a single reaction mixture by contacting, within a single continuous liquid phase, (i) a plurality of target polynucleotides, (ii) a plurality of different sets of particles, wherein each set of particles has a plurality of a polynucleotide-specific capture primer attached thereon, wherein the polynucleotide-specific capture primer differs in sequence between at least two sets of particles, and (iii) a plurality of different polynucleotide-specific solution-phase primers. In some embodiments, the methods further comprise: (b) subjecting the single reaction mixture to a nucleic acid amplification condition, thereby generating from a least one set of particles at least one particle attached to a substantially monoclonal polynucleotide population containing a portion of one of the target polynucleotides from the plurality of target polynucleotides. In some embodiments, the plurality of a target polynucleotide contains an adaptor sequence (e.g., universal adaptor sequence) or lack an adaptor sequence.

[0067] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include a plurality of target polynucleotides which can be isolated from a biological fluid, cell culture, or solid tissue. In some embodiments, the plurality of target polynucleotides is isolated from a biological fluid and the biological fluid comprises

blood, serum, plasma, saliva, sputum, sweat, tears, lavage fluid, amniotic fluid, cerebrospinal fluid, ascites, urine, or semen.

[0068] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include a plurality of different sets of particles, including at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 12, at least 15, at least 20, at least 30, at least 40, at least 50, at least 100, at least 200, at least 300, at least 400, at least 500, at least 1,000, at least 2,000, at least 3,000, at least 4,000, at least 5,000, at least 10,000, or at least 20,000 different sets of particles. In some embodiments, the plurality of different sets of particles is 2 to 1000, or 2 to 900, or 2 to 800, or 2 to 700, or 2 to 600, or 2 to 500, or 2 to 400, or 2 to 300, or 2 to 200, or 2 to 100, or 2 to 50, or 2 to 40, or 2 to 30, or 2 to 20, or 2 to 10, or 5 to 1000, or 5 to 900, or 5 to 800, or 5 to 700, or 5 to 600, or 5 to 500, or 5 to 400, or 5 to 300, or 5 to 200, or 5 to 100, or 5 to 50, or 5 to 40, or 5 to 30, or 5 to 20, or 5 to 10, or 10 to 1000, or 10 to 900, or 10 to 800, or 10 to 700, or 10 to 600, or 10 to 500, or 10 to 400, or 10 to 300, or 10 to 200, or 10 to 100, or 10 to 50 different sets of particles. In some embodiments, each set of particles is a set of microparticles or a set of beads. In some embodiments, each set of particles has a plurality of a polynucleotide-specific capture primers attached thereon. Optionally, the plurality of a polynucleotide-specific capture primer is covalently attached to each set of particles.

[0069] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include at least one polynucleotide-specific capture primer which is capable of hybridizing with at least a portion of a target polynucleotide of the plurality of target polynucleotides. In some embodiments, the 3' region of at least one polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a target polynucleotide of the plurality of target polynucleotides. In some embodiments, each polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a different target polynucleotide of the plurality of target polynucleotides. In some embodiments, the 3' region of each polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a different target polynucleotide of the plurality of target polynucleotides. In some embodiments, at least one polynucleotide-specific capture primer comprises at least one universal sequence. In some embodiments, each polynucleotide-specific capture primer comprises at least one universal sequence. In some embodiments, each universal sequence is

capable of hybridizing with an amplification primer and/or sequencing primer. In some embodiments, the universal sequence does not substantially hybridize with a target polynucleotide. In some embodiments, at least one polynucleotide-specific capture primer comprises at least one unique identifier sequence. In some embodiments, each polynucleotide-specific capture primer comprises at least one unique identifier sequence. In some embodiments, each unique identifier sequence is a sample-specific barcode sequence. In some embodiments, the polynucleotide-specific capture primer of a first set of particles of the plurality of different sets of particles and the polynucleotide-specific capture primer of a second set of particles of the plurality of different sets of particles are capable of hybridizing with different target polynucleotides.

[0070] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include at least one polynucleotide-specific solution-phase primer which is capable of hybridizing with at least a portion of a target polynucleotide of the plurality of target polynucleotides. In some embodiments, a 3' region of at least one polynucleotide-specific solution-phase primer is capable of hybridizing with at least a portion of a target polynucleotide of the plurality of target polynucleotides. In some embodiments, each polynucleotide-specific solution-phase primer is capable of hybridizing with at least a portion of a target polynucleotide of the plurality of target polynucleotides. In some embodiments, a 3' region of each polynucleotide-specific solution-phase primer is capable of hybridizing with at least a portion of a target polynucleotide of the plurality of target polynucleotides. In some embodiments, one of the polynucleotide-specific solution-phase primers is capable of hybridizing with at least a portion of the same target polynucleotide as another polynucleotide-specific solution-phase primer. In some embodiments, two different polynucleotide-specific solution-phase primers are capable of hybridizing with at least a portion of different target polynucleotides. In some embodiments, at least one polynucleotide-specific solution-phase primer comprises at least one universal sequence. In some embodiments, each polynucleotide-specific solution-phase primer comprises at least one universal sequence. In some embodiments, at least one universal sequence is capable of hybridizing with an amplification primer and/or sequencing primer. In some embodiments, the universal sequence does not substantially hybridize with a target polynucleotide. In some embodiments, at least one polynucleotide-specific solution-phase primer comprises at least one unique identifier sequence. In some

embodiments, each polynucleotide-specific solution-phase primer comprises at least one unique identifier sequence. In some embodiments, the unique identifier sequence is a sample-specific barcode sequence. In some embodiments, at least one polynucleotide-specific solution-phase primer is a tailed primer. In some embodiments, each polynucleotide-specific solution-phase primer is a tailed primer. In some embodiments, the polynucleotide-specific solution-phase primer is a reverse amplification primer.

[0071] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids include at least one polynucleotide-specific capture primer and at least one polynucleotide-specific solution-phase primer which hybridize to different portions of the same target polynucleotide. In some embodiments, the portion of the target polynucleotide to which at least one polynucleotide-specific capture primer hybridizes and the portion of the target polynucleotide to which at least one polynucleotide-specific solution-phase primer hybridizes are separated by 10 to 2000 base pairs, or 10 to 1500 base pairs, or 10 to 1000 base pairs, or 10 to 500 base pairs, or 50 to 500 base pairs on the target polynucleotide in its double-stranded form. In some embodiments, the portion of the target polynucleotide to which at least one polynucleotide-specific capture primer hybridizes is on a first strand of the target polynucleotide in its double-stranded form, and the portion of the target polynucleotide to which at least one polynucleotide-specific solution-phase primer hybridizes is on a second strand of the target polynucleotide in its double-stranded form.

[0072] In some embodiments, the nucleic acid amplification condition comprises one primer extension reaction or multiple primer extension reactions. In some embodiments, the nucleic acid amplification condition comprises an isothermal amplification condition or a thermocycling amplification condition. In some embodiments, the single reaction mixture comprises a polymerase and a plurality of nucleotides.

[0073] In some embodiments, the single reaction mixture comprises a recombinase. In some embodiments, the recombinase is uvsX. In some embodiments, the single reaction mixture comprises at least one recombinase accessory protein. In some embodiments, the single reaction mixture comprises at least one recombinase loading protein and/or at least one single-stranded binding protein. In some embodiments, at least one recombinase loading protein is uvsY. In some embodiments, at least one single-stranded binding protein is gp32.

[0074] In some embodiments, the methods, and related compositions, systems, kits and apparatus, for amplifying nucleic acids further comprise sequencing at least one substantially monoclonal polynucleotide population. In some embodiments, the method comprises sequencing a plurality of substantially monoclonal polynucleotide population. In some embodiments, the method comprises sequencing each substantially monoclonal polynucleotide population. In some embodiments, the sequencing comprises contacting at least one sequencing primer to at least one substantially monoclonal polynucleotide population. In some embodiments, the sequencing comprises contacting at least one sequencing primer to each substantially monoclonal polynucleotide population. In some embodiments, at least one sequencing primer is capable of hybridizing to a portion of a polynucleotide of a substantially monoclonal polynucleotide population. In some embodiments, at least one sequencing primer is a universal sequencing primer. In some embodiments, the universal sequence does not substantially hybridize with a target polynucleotide.

[0075] In some embodiments, at least one particle attached to a substantially monoclonal polynucleotide population is deposited on a support having at least one reaction site operatively coupled to a sensor. In some embodiments, each particle attached to a substantially monoclonal polynucleotide population is deposited on a support having at least one reaction site operatively coupled to a sensor. In some embodiments, at least one particle attached to a substantially monoclonal polynucleotide population is deposited on a reaction site of a support having an array of reaction sites. In some embodiments, each particle attached to a substantially monoclonal polynucleotide population is deposited on a reaction site of a support having an array of reaction sites. In some embodiments, the individual reaction sites in the array are operatively coupled to at least one sensor. In some embodiments, each individual reaction site in the array is operatively coupled to a different sensor. In some embodiments, the method comprises sequencing at least one substantially monoclonal polynucleotide population, and wherein the sequencing is conducted at a reaction site. In some embodiments, the sequencing comprises detecting a nucleotide incorporation byproduct using the at least one sensor operatively coupled to the reaction site. In some embodiments, the nucleotide incorporation byproduct is selected from hydrogen ions, hydroxyl ions, pyrophosphate, charge transfer, and heat. In some embodiments, the sensor detects a change in pH. In some embodiments, the sensor comprises a field effect transistor (FET). In some embodiments, the sensor comprises a chemical field effect transistor

(chemFET). In some embodiments, the sensor comprises an ion-sensitive field effect transistor (ISFET).

[0076] In some embodiments, the single continuous liquid phase does not provide compartmentalization. In some embodiments, the single continuous liquid phase lacks an oil and water emulsion. In some embodiments, the single continuous liquid phase is an aqueous phase.

[0077] In some embodiments, the plurality of target polynucleotides comprises chromatin, genomic DNA, RNA, fragmented DNA, fragmented RNA, amplified DNA, amplified RNA, or nucleic acid molecules from an RNA or DNA library (where the library molecules optionally include at least one adaptor sequence, e.g., universal adaptor sequence). In some embodiments, the plurality of target polynucleotides comprises mRNA, miRNA, or tRNA. In some embodiments, the plurality of target polynucleotides is obtained from a healthy subject, or from a subject having a disease, or from a subject suspected of having a disease. In some embodiments, the plurality of target polynucleotides is obtained from a subject having a disease or from a subject suspected of having a disease. Optionally, the disease is a genetic disease or an infectious disease. In some embodiments, the disease is an infectious disease caused by an infectious agent selected from a bacterium, a mycobacterium, a virus, a fungus, and a parasite. In some embodiments, the disease is sepsis, pneumonia, MRSA, genitourinary tract infection, tuberculosis, hepatitis, HIV, candidiasis, or malaria. In some embodiments, the disease includes water-borne or food-borne diseases from water or food contaminated with one or more pathogens. In some embodiments, the pathogen itself causes the disease or toxins produced by the pathogen cause the disease. In some embodiments, the disease can be caused by *Campylobacter*, *Salmonella*, *Shigella*, *E. coli*, *Listeria*, *Staphylococcus*, *Clostridium*, and/or novovirus.

[0078] In some embodiments, the present disclosure provides methods, compositions, systems and kit, for amplifying nucleic acids which are depicted in Figures 1A and B, comprising: (a) forming a single reaction mixture by contacting, within a single continuous liquid phase, (i) a plurality of target polynucleotides, which includes a first target polynucleotide **(10)** which optionally includes or lacks a universal adaptor sequence, (ii) a plurality of sets of particles, including a first set of particles having a plurality of a first polynucleotide-specific capture primer attached thereon **(40)**, which includes a first particle **(41)** attached with a first universal sequence region **(42)** and a first polynucleotide-specific capture region **(43)** and, (iii) a

plurality of sets of particles, including a second set of particles having a plurality of a second polynucleotide-specific capture primer attached thereon **(50)**, which includes a second particle **(51)** attached with a second universal sequence region **(52)** and a second polynucleotide-specific capture region **(53)**, wherein the first **(42)** and second **(52)** universal sequence regions (on the first and second sets of particles, respectively) have the same sequence or different sequences, wherein the first **(43)** and second **(53)** polynucleotide-specific capture primer sequence regions (on the first and second sets of particles, respectively) have the same sequence or different sequences, and (iv) a plurality of a first polynucleotide-specific solution-phase primers, including a first polynucleotide-specific solution-phase primer **(30)** having a first polynucleotide-specific region **(31)** and first universal sequence regions **(32)** and **(33)**, (v) a plurality of a second polynucleotide-specific solution phase primer, including a second polynucleotide-specific solution-phase primer **(20)** having a second polynucleotide-specific region **(21)** and second universal sequence regions **(22)** and **(23)**, wherein the first **(32)** and **(33)**, and the second **(22)** and **(23)**, universal sequence regions (of the first and second polynucleotide-specific solution-phase primers, respectively) have the same sequence or different sequences, and wherein the first **(31)**, and the second **(21)**, polynucleotide-specific regions (of the first and second polynucleotide-specific solution-phase primers, respectively) have the same sequence or different sequences. In some embodiments, the single reaction mixture further comprises a recombinase (e.g., uvsX or recA), at least one recombinase loading protein (e.g., uvsY), at least one single-stranded binding protein (e.g., gp32 protein or SSB), helicase (e.g., uvsW protein), topoisomerase, an inorganic phosphatase (e.g., from yeast), PEG and/or trehalose. In some embodiments, the methods further comprise: (b) subjecting the single reaction mixture to a nucleic acid amplification condition, for example an isothermal amplification condition (Figure 1A). In some embodiments, the nucleic acid amplification condition generates, a set of particles attached to substantially monoclonal polynucleotides **(60)** and **(70)** which includes a portion of the target polynucleotide (Figure 1B). In some embodiments, the nucleic acid amplification condition generates, from the first set of particles, at least one particle **(41)** attached to a first substantially monoclonal polynucleotide population containing a first portion **(11)** of one of the target polynucleotides from the plurality of target polynucleotides. In some embodiments, the first substantially monoclonal polynucleotide population also includes a first universal sequence region **(42)**, and a first universal sequence region **(32)** and **(33)**. In some embodiments, the nucleic acid amplification

condition generates, from the second set of particles, at least one particle **(51)** attached to a second substantially monoclonal polynucleotide population containing a second portion **(12)** of one of the target polynucleotides from the plurality of target polynucleotides. In some embodiments, the second substantially monoclonal polynucleotide population also includes a second universal sequence region **(52)**, and a second universal sequence region **(22)** and **(33)**. In some embodiments, the monoclonal polynucleotide population containing a first portion **(11)** of one of the target polynucleotides, and the monoclonal polynucleotide population containing a second portion **(12)** of one of the target polynucleotides, can be amplified from different regions of the same target polynucleotide (e.g., as shown in Figure 1A), or can be amplified from different (separate) target polynucleotides.

[0079] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatuses, comprising preparing a set of supports (e.g., particles or beads) having a plurality of a polynucleotide-specific capture primers attached thereon. In some embodiments, the plurality of polynucleotide-specific capture primers can be modified to carry a first chemical compound to permit attachment of their 5' or 3' ends to a plurality of supports, where the surface of the supports can optionally be modified to carry a second chemical compound that will react and bind with the first chemical compound that is carried on the polynucleotide-specific capture primers. In an alternative embodiment, the starting material can include supports (e.g., particles or beads) that are already attached to a plurality of universal primer sequences, where the capture primers are enzymatically ligated to the plurality of polynucleotide-specific capture primers. Optionally, the ligation reaction can be performed with a soluble fusion primer that serves as a splint molecule to increase the yield of ligation products. A schematic of the splint-ligation method is shown in Figure 2, which depicts a ligation reaction using a single bead attached with a single universal primer sequences, but the skilled artisan will appreciate that the ligation reaction can be performed using a plurality of supports, where substantially each support is attached with a plurality of universal primer sequences. The skilled artisan will also appreciate that the 5' and 3' ends of the nucleic acid molecules depicted in Figure 2 (e.g., **(90)**, **(100)** and **(110)**) can be reversed. For example, the 5' ends of nucleic acid molecule **(110)** is oriented at the left side of Figure 2, and the 3' ends of nucleic acid molecule **(90)** and **(100)** are oriented at the left side of Figure 2. In some embodiments, the ligation reaction includes: contacting (i) a plurality of supports **(80)** which are

attached with a plurality of universal primer sequences (90), and (ii) a plurality of polynucleotide-specific capture primers (100), and (iii) a plurality of solution-phase fusion primers (110), under conditions that are suitable for binding one portion of the solution-phase fusion primers (110) to the plurality of universal primer sequences (90), and binding another portion of the solution-phase fusion primers (110) to the polynucleotide-specific capture primers (100), and under conditions that are suitable for ligating together one end of the universal primer sequences (90) to one end of the polynucleotide-specific capture primers (100), thereby generating a plurality of supports (80) having a plurality of ligation products (120) attached thereon. In some embodiments, the solution-phase fusion primers (110) include a region that can hybridize to at least a portion of the universal primer sequences (90). In some embodiments, the solution-phase fusion primers (110) also include a region that can hybridize to at least a portion polynucleotide-specific capture primers (100). In some embodiments, the 5' end of the polynucleotide-specific capture primers (100) can include a phosphate group to permit ligation to the 3' end of the universal primer sequences (90). In some embodiments, the 3' end of the polynucleotide-specific capture primers (100) can include a hydroxyl group to permit ligation to the 5' end of the universal primer sequences (90). In some embodiments, the ligation products (120) include the universal primer sequences (90) joined (e.g., covalently joined) to the polynucleotide-specific capture primers (100). In some embodiments, the ligation reaction can be conducted in a way that converts many of the universal primer sequences (90) to ligation products (120), and leaving the remainder of the universal primer sequences (90) un-ligated. For example, about 50-75%, or about 75-85%, or about 85-95%, or about 95-99%, of the universal primer sequences (90) can be converted to ligation products (120). In some embodiments, the ligation reaction can be conducted in a way that converts only a small percent of the universal primer sequences (90) to ligation products (120). For example, about 1-5%, about 5-10%, or about 10-25%, or about 25-35%, or about 35-50%, of the universal primer sequences (90) are converted to ligation products (120), and leaving the remainder of the universal primer sequences (90) un-ligated. The ability to produce supports having a limited percentage of the universal primer sequences (90) that are converted to ligation products (120), can act to control the concentration of different primers (e.g., (90) and (120)) in an amplification reaction, which can advantageously be used to reduce formation of primer-dimers. In some embodiments, any nucleic acid amplification reaction of the present teachings can be performed using supports

having any percentage (e.g., 1-99%) of the universal primer sequences **(90)** that are converted to ligation products **(120)**, where the converted to ligation products **(120)** act as a fusion primer to amplify a target polynucleotide, and the resulting amplicons (optionally in the presence of solution-phase reverse primers) can be amplified using the un-ligated universal primer sequences **(90)**. In some embodiments, the supports having a limited percentage of the universal primer sequences **(90)** that are converted to ligation products **(120)**, can be used to increase the yield of supports attached to substantially monoclonal polynucleotides.

[0080] In some embodiments, the disclosure relates generally to compositions, and related methods, systems, kits and apparatuses, comprising: (i) a plurality of target polynucleotides, and (ii) a plurality of different sets of particles, wherein each set of particles has a plurality of a polynucleotide-specific capture primer attached thereon, wherein the polynucleotide-specific capture primer differs in sequence between at least two sets of particles, and wherein at least one of the polynucleotide-specific capture primers hybridizes with at least a portion of at least one of the target polynucleotides. In some embodiments, the compositions, and related methods, systems, kits and apparatuses, further comprise: a uvsX recombinase. In some embodiments, the compositions, and related methods, systems, kits and apparatuses, further comprise: a uvsY recombinase loading protein. In some embodiments, the compositions, and related methods, systems, kits and apparatuses, further comprise: a gp32 single stranded binding protein. In some embodiments, the compositions, and related methods, systems, kits and apparatuses, further comprise: a polymerase. In some embodiments, the compositions, and related methods, systems, kits and apparatuses, further comprise: a plurality of nucleotides. In some embodiments, the compositions, and related methods, systems, kits and apparatuses, further comprise: inorganic phosphatase (e.g., from yeast), PEG and/or trehalose.

[0081] In some embodiments, the disclosure relates generally to compositions, and related methods, systems, kits and apparatuses, comprising: (i) a plurality of target polynucleotides, and (ii) a plurality of different sets of particles, wherein each set of particles has a plurality of a polynucleotide-specific capture primer attached thereon, wherein the polynucleotide-specific capture primer differs in sequence between at least two sets of particles, and wherein at least one of the polynucleotide-specific capture primers hybridizes with at least a portion of at least one of the target polynucleotides, and further comprise any one or any combination of a uvsX recombinase, a uvsY recombinase loading protein, a gp32 single stranded binding protein, a

polymerase, plurality of nucleotides, inorganic phosphatase (e.g., from yeast), PEG and/or trehalose.

[0082] In some embodiments, the compositions, and related methods, systems, kits and apparatus, comprise a plurality of different sets of particles, wherein each set of particles has a plurality of a polynucleotide-specific capture primer attached thereon, wherein the polynucleotide-specific capture primer differs in sequence between at least two sets of particles.

[0083] In some embodiments, the plurality of different sets of particles is at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 12, at least 15, at least 20, at least 30, at least 40, at least 50, at least 100, at least 200, at least 300, at least 400, at least 500, at least 1,000, at least 2,000, at least 3,000, at least 4,000, at least 5,000, at least 10,000, or at least 20,000 different sets of particles. In some embodiments, the plurality of different sets of particles is 2 to 1000, or 2 to 900, or 2 to 800, or 2 to 700, or 2 to 600, or 2 to 500, or 2 to 400, or 2 to 300, or 2 to 200, or 2 to 100, or 2 to 50, or 2 to 40, or 2 to 30, or 2 to 20, or 2 to 10, or 5 to 1000, or 5 to 900, or 5 to 800, or 5 to 700, or 5 to 600, or 5 to 500, or 5 to 400, or 5 to 300, or 5 to 200, or 5 to 100, or 5 to 50, or 5 to 40, or 5 to 30, or 5 to 20, or 5 to 10, or 10 to 1000, or 10 to 900, or 10 to 800, or 10 to 700, or 10 to 600, or 10 to 500, or 10 to 400, or 10 to 300, or 10 to 200, or 10 to 100, or 10 to 50 different sets of particles.

[0084] In some embodiments, each set of particles is a set of microparticles or a set of beads. In some embodiments, each set of particles has a plurality of a polynucleotide-specific capture primers attached thereon. Optionally, the polynucleotide-specific capture primers are covalently attached to each set of particles. In some embodiments, at least one polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a target polynucleotide of a plurality of target polynucleotides. In some embodiments, the 3' region of at least one polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a target polynucleotide of a plurality of target polynucleotides. In some embodiments, each polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a different target polynucleotide of a plurality of target polynucleotides. In some embodiments, the 3' region of each polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a different target polynucleotide of the plurality of target polynucleotides. In some embodiments, at least one polynucleotide-specific capture primer comprises at least one universal sequence. In some embodiments, each polynucleotide-specific capture primer

comprises at least one universal sequence. In some embodiments, each universal sequence is capable of hybridizing with an amplification primer and/or sequencing primer. In some embodiments, the universal sequence does not substantially hybridize with a target polynucleotide. In some embodiments, at least one polynucleotide-specific capture primer comprises at least one unique identifier sequence. In some embodiments, each polynucleotide-specific capture primer comprises at least one unique identifier sequence. In some embodiments, each unique identifier sequence is a sample-specific barcode sequence. In some embodiments, the polynucleotide-specific capture primer of a first set of particles of the plurality of different sets of particles and the polynucleotide-specific capture primer of a second set of particles of the plurality of different sets of particles are capable of hybridizing with different target polynucleotides.

[0085] In some embodiments, the compositions, and related methods, systems, kits and apparatus, comprise polynucleotides isolated from a biological fluid, cell culture, or solid tissue. In some embodiments, the plurality of target polynucleotides comprises polynucleotides isolated from a biological fluid and the biological fluid comprises blood, serum, plasma, saliva, sputum, sweat, tears, lavage fluid, amniotic fluid, cerebrospinal fluid, ascites, urine, or semen.

[0086] In some embodiments, the compositions, and related methods, systems, kits and apparatus, comprise particles which can be deposited on a support. In some embodiments, the support has at least one reaction site operatively coupled to a sensor. In some embodiments, at least one set of particles is deposited on a reaction site of a support having an array of reaction sites. In some embodiments, each set of particles is deposited on a reaction site of a support having an array of reaction sites. In some embodiments, the individual reaction sites in the array are operatively coupled to at least one sensor. In some embodiments, each individual reaction site in the array is operatively coupled to a different sensor. In some embodiments, a sensor is configured to detect a nucleotide incorporation byproduct. In some embodiments, the nucleotide incorporation byproduct is selected from hydrogen ions, hydroxyl ions, pyrophosphate, charge transfer, and heat. In some embodiments, a sensor is configured to detect a change in pH.

[0087] As used herein the terms “amplify”, “amplifying”, “amplification” and other related terms include producing multiple copies of an original biomolecule. In some embodiments, nucleic acid amplification produces multiple copies of an original polynucleotide (e.g., target

polynucleotide), where the copies comprise a template sequence, or the copies comprise a sequence that is substantially identical to a template sequence.

[0088] As used herein the terms “hybridize”, “hybridizing”, “hybridization” and other related terms include hydrogen bonding between two different nucleic acids, or between two different regions of a nucleic acid, to form a duplex nucleic acid. Hybridization can comprise Watson-Crick or Hoogsteen binding to form a duplex nucleic acid. The two different nucleic acids, or the two different regions of a nucleic acid, may be complementary, or partially complementary. The complementary base pairing can be the standard A-T or C-G base pairing, or can be other forms of base-pairing interactions. Duplex nucleic acids can include mismatched base-paired nucleotides. Complementary nucleic acid strands need not hybridize with each other across their entire length.

[0089] In some embodiments, the disclosure relates generally to methods for amplifying nucleic acids, and related compositions, systems, kits and apparatuses, comprising conditions that are suitable for nucleic acid hybridization and/or for washing conditions, which include parameters such as salts, buffers, pH, temperature, GC% content of the polynucleotide and primers, and/or time. For example, conditions suitable for hybridizing or washing nucleic acids (e.g., polynucleotides and primers) can include hybridization solutions having sodium salts, such as NaCl, sodium citrate and/or sodium phosphate. In some embodiments, hybridization or wash solutions can include formamide (e.g., about 10-75%) and/or sodium dodecyl sulfate (SDS) (e.g., about 0.01-0.7%). In some embodiments, a hybridization solution can be a stringent hybridization solution which can include any combination of formamide (e.g., about 50%), 5 x SSC (e.g., about 0.75 M NaCl and about 0.075 M sodium citrate), sodium phosphate (e.g., about 50 mM at about pH 6.8), sodium pyrophosphate (e.g., about 0.1%), 5X Denhardt's solution, SDS (e.g., about 0.1%), and/or dextran sulfate (e.g., about 10%). In some embodiments, the hybridization or washing solution can include BSA (bovine serum albumin). In some embodiments, hybridization or washing can be conducted at a temperature range of about 15-25°C, or about 25-35°C, or about 35-45°C, or about 45-55°C, or about 55-65°C, or about 65-75°C, or about 75-85°C, or about 85-95°C, or about 95-99°C, or higher.

[0090] In some embodiments, hybridization or washing can be conducted for a time range of about 1-10 minutes, or about 10-20 minutes, or about 20-30 minutes, or about 30-40 minutes, or about 40-50 minutes, or about 50-60 minutes, or longer.

[0091] In some embodiments, hybridization or wash conditions can be conducted at a pH range of about 5-10, or about pH 6-9, or about pH 6.5-8, or about pH 6.5-7.

[0092] Methods for nucleic acid hybridization and washing are well known in the art. For example, thermal melting temperature (T_m) for nucleic acids can be a temperature at which half of the nucleic acid strands are double-stranded and half are single-stranded under a defined condition. In some embodiments, a defined condition can include ionic strength and pH in an aqueous reaction condition. A defined condition can be modulated by altering the concentration of salts (e.g., sodium), temperature, pH, buffers, and/or formamide. Typically, the calculated thermal melting temperature can be at about 5-30° C below the T_m , or about 5-25° C below the T_m , or about 5-20° C below the T_m , or about 5-15° C below the T_m , or about 5-10° C below the T_m . Methods for calculating a T_m are well known and can be found in Sambrook (1989 in “Molecular Cloning: A Laboratory Manual”, 2nd edition, volumes 1-3; Wetmur 1966, J. Mol. Biol., 31:349-370; Wetmur 1991 Critical Reviews in Biochemistry and Molecular Biology, 26:227-259). Other sources for calculating a T_m for hybridizing or denaturing nucleic acids include OligoAnalyze (from Integrated DNA Technologies) and Primer3 (distributed by the Whitehead Institute for Biomedical Research).

[0093] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatuses, comprising amplifying nucleic acids in the presence of one or more supports (e.g., particles or beads) having a surface. In some embodiments, the term “surface” can be an outer or top-most layer or boundary of an object. In some embodiments, a surface can be interior to the boundary of an object. In some embodiments, a surface can be a planar surface, as well as concave, convex, or any combination thereof. In some embodiments, a surface can be a bead, particle, microparticle, sphere, filter, flowcell, well, microwell, groove, channel reservoir, gel or inner wall of a capillary. In some embodiments, a surface includes the inner walls of a capillary, a channel, a well, microwell, groove, channel, reservoir. In some embodiments, a surface can include texture (e.g., etched, cavitated, pores, three-dimensional scaffolds or bumps). In some embodiments, a surface can be porous, semi-porous or non-porous. In some embodiments, particles can have a shape that is spherical, hemispherical, cylindrical, barrel-shaped, toroidal, rod-like, disc-like, conical, triangular, cubical, polygonal, tubular, wire-like or irregular. In some embodiments, a surface can be made from any material, including glass, borosilicate glass, silica, quartz, fused quartz, mica,

polyacrylamide, plastic polystyrene, polycarbonate, polymethacrylate (PMA), polymethyl methacrylate (PMMA), polydimethylsiloxane (PDMS), silicon, germanium, graphite, ceramics, silicon, semiconductor, high refractive index dielectrics, crystals, gels, polymers, or films (e.g., films of gold, silver, aluminum, or diamond). In some embodiments, a surface can be magnetic or paramagnetic. In some embodiments, a surface can be paramagnetic beads (particle) attached with streptavidin, for example Dynabeads™ M-270 (from Invitrogen, Carlsbad, CA). A bead or particle can have an iron core, or comprise a hydrogel or agarose (e.g., Sepharose™). In some embodiments, the surface (including interior scaffolds of a bead or particle) can be attached with a plurality of a capture primer. A surface can be coated with an acrylamide, carboxylic or amine compound for attaching a nucleic acid (e.g., a capture primer). In some embodiments, an amino-modified nucleic acid (e.g., primer) can be attached to a surface that is coated with a carboxylic acid. In some embodiments, an amino-modified nucleic acid can be reacted with ethyl (dimethylaminopropyl) carbodiimide (EDC) or EDAC for attachment to a carboxylic acid coated surface (with or without N-hydroxysuccinimide (NHS)). A capture primer can be immobilized to an acrylamide compound coating on a surface. The particles can be coated with an avidin-like compound (e.g., streptavidin) for binding biotinylated nucleic acids.

[0094] In some embodiments, the disclosure relates generally to methods, as well as related systems, compositions, kits and apparatuses, comprising amplifying nucleic acids in the presence of particles, microparticles or beads. One skilled in the art will appreciate that the methods, as well as related systems, compositions, kits and apparatuses, can include a first, and optionally a second, third, fourth, fifth, sixth, seventh, eighth, ninth, tenth, eleventh, or more different sets of particles. In some embodiments, the nucleic acid amplification reaction includes 1-25, 25-50, 50-75, 75-100, 100-500, 500-1,000, 1,000-10,000, or 10^4 - 10^{10} or more sets of beads or particles. In some embodiments, different capture primers are attached (e.g., immobilized) to the different sets of particles. For example, a first set of particles is attached with a plurality of a first capture primer, and a second set of particles is attached with a plurality of a second capture primer, and so on with the other sets of particles. In some embodiments, the particles can be attached with a plurality of one capture primers having the same sequence, or can be attached a plurality of two or more different capture primers having different sequences. The capture primer can selectively bind (hybridize) to at least a portion of the target polynucleotide or an adaptor sequence.

[0095] In some embodiments, the plurality of particles can be solid, or can have an outer surface and an interior surface. The plurality of particles can be porous, semi porous or non-porous. The plurality of particles can have cavitation or pores, or can include three-dimensional scaffolds. In some embodiments, the plurality of particles can be Ion Sphere™ particles (from Ion Torrent, part of Thermo Fisher Scientific).

[0096] In some embodiments, the plurality of a particle comprises a polymer material. For example, the plurality of a particle comprises a gel, hydrogel or acrylamide polymers. In some embodiments, the plurality of particles can have any shape that is spherical, hemispherical, cylindrical, barrel-shaped, toroidal, rod-like, disc-like, conical, triangular, cubical, polygonal, tubular, wire-like or irregular.

[0097] In some embodiments, the particles can be any size that can fit into a reaction chamber. For example, the particles can be small enough to fit one particle in a reaction chamber. In some embodiments, the particles can be small enough so that more than one particle can fit in a reaction chamber. In some embodiments, the smallest cross-sectional length of a particle (e.g., diameter) can be about 50 microns or less, or about 10 microns or less, or about 3 microns or less, approximately 1 micron or less, approximately 0.5 microns or less, e.g., approximately 0.1, 0.2, 0.3, or 0.4 microns, or smaller (e.g., under 1 nanometer, about 1-10 nanometer, about 10-100 nanometers, or about 100-500 nanometers).

[0098] In some embodiments, the particles can be attached with a plurality of at least 1,000 oligonucleotide primers, or about 1,000 – 10,000 oligonucleotide primers, or about, 10,000 – 50,000 oligonucleotide primers, or about 50,000 – 75,000 oligonucleotide primers, or about 75,000 – 100,000 oligonucleotide primers, or more.

[0099] In some embodiments, the exterior surface and/or the interior scaffold of a particle can be attached with one or more capture primers. Optionally, the capture primer includes a universal priming sequence or site. Optionally, the capture primers can include any or any combination of an amplification primer sequence, a sequencing primers sequence, unique identifier sequence and/or a source identifier sequence.

[00100] In some embodiments, a particle surface, and optionally interior scaffold, can be coated with an acrylamide, carboxylic or amine compound for attaching a nucleic acid (e.g., capture primer). In some embodiments, an amino-modified capture primer can be attached to a particle surface that is coated with a carboxylic acid. In some embodiments, an amino-modified

capture primer can be reacted with ethyl (dimethylaminopropyl) carbodiimide (EDC) or EDAC for attachment to a carboxylic acid coated surface (with or without N-hydroxysuccinimide (NHS)). A capture primer can be immobilized to an acrylamide compound coating on a particle surface. Particles can be coated with an avidin-like compound (e.g., streptavidin) for binding biotinylated capture primers.

[00101] In some embodiments, the disclosure relates generally to methods, as well as related systems, compositions, kits and apparatuses, comprising amplifying nucleic acids using one or more capture primers attached to a support, including a particle or planar-like surface. In some embodiments, the methods, as well as related systems, compositions, kits and apparatuses, comprise at least a first set of particles attached to a plurality of a first capture primer. In some embodiments, the methods, as well as related systems, compositions, kits and apparatuses, additionally include a second particle attached to a plurality of a second capture primer. In some embodiments, the compositions, as well as related systems, methods, kits and apparatuses, comprise additional capture primers, including a third, fourth, fifth, sixth, seventh, eighth, ninth, tenth, eleventh, or more different capture primers. In some embodiments, the nucleic acid amplification reaction includes 1-25, 25-50, 50-75, 75-100, 100-500, 500-1,000, 1,000-10,000, or 10^4 - 10^{10} or more different sets of beads or particles where each set is attached with capture primers that differ in sequence than the capture primers attached to other sets of beads or particles. In some embodiments, the capture primers of one set have a different nucleotide sequence compared to the capture primers of a different set. In some embodiments, the capture primers comprise polymers of deoxyribonucleotides, ribonucleotides, and/or analogs thereof. In some embodiments, the capture primers comprise naturally-occurring, synthetic, recombinant, cloned, amplified, or unamplified forms. In some embodiments, the capture primers comprise DNA, cDNA, RNA, chimeric RNA/DNA, or nucleic acid analogs. In some embodiments, the capture primers comprise single-stranded oligonucleotides. In some embodiments, the capture primers comprise a random or degenerate sequence. In some embodiments, at least one portion of the capture primer comprises a sequence that can selectively bind (hybridize) to at least one portion of the target polynucleotide. For example, the capture primer includes a gene-specific or target-specific sequence, and hybridizes to one strand of the target polynucleotide. In some embodiments, at least one portion of the capture primer hybridizes to an adaptor joined to the target polynucleotide, or the junction between the adaptor and target polynucleotide. In some

embodiments, at least one portion of the capture primers comprises a sequence that can hybridize with at least one portion of a fusion primer. In some embodiments, at least one portion of the capture primers comprises a sequence that is identical or is partially or fully complementary to a portion of a target polynucleotide, an adaptor, or a fusion primer. For example, the 3' region of the capture primers comprises a sequence that is identical or is complementary to a portion of a target polynucleotide, an adaptor, or a fusion primer. A "polynucleotide-specific capture primer" refers to a capture primer that is capable of hybridizing to a portion of a target polynucleotide under conditions suitable for primer extension, amplification, and/or sequencing. Optionally, the capture primer includes at least one universal sequence (amplification or sequencing primer sequence) and/or at least one unique identifier sequence (e.g., a sample-specific barcode sequence).

[00102] In some embodiments, the 5' or 3' end of the capture primer can be modified for attachment to the support. For example, a 5' or 3' end can be modified to include an amino group that can bind to a carboxylic acid compound on the support. In another example, a 5' end can include a phosphate group for reacting with an amine-coated surface in the presence of a carbodiimide (e.g., water soluble carbodiimide).

[00103] In some embodiments, the capture primers comprise forward amplification primers. In some embodiments, the capture primers comprise reverse amplification primers.

[00104] In some embodiments, the 3' end of the capture primer is extendible in a primer extension reaction. Optionally, the 3' end of the capture primer includes a 3'OH group. In some embodiments, the capture primer has a blocking moiety that prevents extension in a primer extension reaction.

[00105] In some embodiments, the capture primers can be any length, including about 2-100 nucleotides, or about 5-10 nucleotides, or about 10-25 nucleotides, or about 25-40 nucleotides, or about 40-55 nucleotides, or about 55-70 nucleotides, or about 70-85 nucleotides, or about 85-100 nucleotides, or longer.

[00106] In some embodiments, the capture primers include at least one linkage or base that is resistant to degradation by an exonuclease or endonuclease. For example, the fusion primers and reverse amplification primers can include at least one phosphorothioate linkage or a 3'-3' end linkage for exonuclease resistance, or at least one 2' fluoro or 2'O-methyl modification

for endonuclease resistance. In some embodiments, the capture primer includes at least one phosphorothiolate, phosphorothioate, and/or phosphoramidate linkage.

[00107] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatuses, comprising amplifying nucleic acids that optionally can include a solution-phase primer (e.g., soluble primer). In some embodiments, at least one portion of a solution-phase primer comprises a sequence that can selectively bind (hybridize) with at least one portion of the target polynucleotide, or an adaptor joined to the target polynucleotide, a capture primer, or a fusion primer. In some embodiments, at least one portion of the solution-phase primer comprises a sequence that is substantially identical or is complementary to a portion of a target polynucleotide, an adaptor, a capture primer, or a fusion primer. In some embodiments, the solution-phase primer hybridizes to at least a portion of one strand of the target polynucleotide (e.g., gene-specific, target-specific, or polynucleotide-specific), or hybridizes to at least a portion of an amplicon generated from the target polynucleotide. In some embodiments, at least a portion of the solution-phase primer can be partially or fully complementary to a portion of the target polynucleotide or to the nucleic acid adaptor or to a junction between the target polynucleotide and adaptor. A “polynucleotide-specific solution-phase primer” is capable of hybridizing to a portion of a target polynucleotide under conditions suitable for primer extension, amplification, and/or sequencing.

[00108] In some embodiments, the solution-phase primer comprises single-stranded oligonucleotides. In some embodiments, the solution-phase primer is not attached to any support (e.g., a particle or planar-like surface). Optionally, the solution-phase primer comprises a reverse amplification primer. Optionally, the solution-phase primer comprises a forward amplification primer. Optionally, the solution-phase primer includes a universal priming sequence or site. Optionally, the solution-phase primers can include any one or any combination of an amplification primer sequence, a sequencing primers sequence, unique identifier sequence and/or a source identifier sequence. Optionally, the solution-phase primer includes a binding partner. Optionally, the binding partner comprises biotin. Optionally, the 5' end of the solution-phase primer can include a sequence that is not contained in, or is not complementary to, a sequence in the target polynucleotide. For example, the solution-phase primer can be a tailed primer.

[00109] In some embodiments, the solution-phase primer comprises polymers of deoxyribonucleotides, ribonucleotides, and/or analogs thereof. In some embodiments, the

solution-phase primer comprises naturally-occurring, synthetic, recombinant, cloned, amplified, or unamplified forms. In some embodiments, the solution-phase primer comprises DNA, cDNA, RNA, chimeric RNA/DNA, or nucleic acid analogs. In some embodiments, the solution-phase primer comprises a random or degenerate sequence.

[00110] In some embodiments, the 3' end of the solution-phase primer is extendible in a primer extension reaction. Optionally, the 3' end of the solution-phase primer includes a 3'OH group. In some embodiments, the solution-phase primer has a blocking moiety that prevents extension in a primer extension reaction.

[00111] In some embodiments, the solution-phase primer can be any length, including about 2-100 nucleotides, or about 5-10 nucleotides, or about 10-25 nucleotides, or about 25-40 nucleotides, or about 40-55 nucleotides, or about 55-70 nucleotides, or about 70-85 nucleotides, or about 85-100 nucleotides, or longer.

[00112] In some embodiments, the solution-phase primers include at least one linkage or base that is resistant to degradation by an exonuclease or endonuclease. For example, the fusion primers and reverse amplification primers can include at least one phosphorothioate linkage or a 3'-3' end linkage for exonuclease resistance, or at least one 2' fluoro or 2'O-methyl modification for endonuclease resistance.

[00113] In some embodiments, the solution-phase primer comprises a binding partner or affinity moiety (e.g., biotin) for affinity-based enrichment of the amplified polynucleotides. Optionally, the solution-phase primer includes at least one universal sequence (amplification or sequencing primer sequence) and/or at least one unique identifier sequence (e.g., a sample-specific barcode sequence).

[00114] In some embodiments, the 5' or 3' region of the solution-phase primer includes a sequence that is substantially non-complementary to a portion of the target polynucleotide or to a portion of any adaptor (e.g., a tailed primer).

[00115] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatuses, comprising amplifying nucleic acids, which optionally includes using a fusion primer. The fusion primer can be attached to a support or can be a solution-phase primer. The fusion primer can include a primer-extendible 3' end, or can have a moiety that blocks primer extension. The fusion primer can include two or more regions, where each region provides a different function. In some embodiments, at least a portion of the

fusion primer can be partially or fully complementary to a portion of the target polynucleotide or a portion of the amplicon generated from the target polynucleotide or the capture primer.

[00116] For example, a solution-phase fusion primer can include (i) a 3' region that can hybridize to one strand of a target polynucleotide, and (ii) a 5' region that can carry a universal sequence, an amplification primer sequence, a sequencing primer sequence, a restriction enzyme recognition sequence, a promoter sequence, a cleavable site, or a sample-specific barcode sequence. Optionally, the 5' region does not substantially hybridize to the target polynucleotide. Optionally, the 5' region of substantially all of the solution phase fusion primer can include at least one universal sequence. In some embodiments, this type of fusion primer includes a solution-phase, tailed primer that can be used to amplify a target polynucleotide.

[00117] In another example, the fusion primer can be attached to a support (e.g., particle, bead or flowcell), and can include (i) a 3' region that can hybridize to one strand of a target polynucleotide, and (ii) a 5' region that can carry a universal sequence, an amplification primer sequence, a sequencing primer sequence, a restriction enzyme recognition sequence, a promoter sequence, a cleavable site, or a sample-specific barcode sequence. Optionally, the 5' region does not substantially hybridize to the target polynucleotide. Optionally, the 5' region of substantially all of this type of immobilized fusion primer can include at least one universal sequence. In some embodiments, this type of fusion primer includes a polynucleotide-specific capture primer that is attached at its 5' end to a support, and can be used to amplify and attach a target polynucleotide to the support.

[00118] In yet another example, the fusion primer can serve as a splint molecule (e.g., **(110)** in Figure 2) by hybridizing to two different nucleic acid molecules. For example, (i) one region of the fusion primer can hybridize to a first nucleic acid molecule, and (ii) a second region can hybridize to a second nucleic acid molecule. In some embodiments, (i) the first region of the split primer can hybridize to one strand of a target polynucleotide, and (ii) the second region that can carry a universal sequence, an amplification primer sequence, a sequencing primer sequence, a restriction enzyme recognition sequence, a promoter sequence, a cleavable site, or a sample-specific barcode sequence. Optionally, the second region of the splint primer does not substantially hybridize to the target polynucleotide. Optionally, the second region of substantially all of the splint primers can include at least one universal sequence. The splint

fusion primer can be a solution-phase primer, or can be attached to a support. In some embodiments, the splint fusion primer can be used in a ligation reaction to increase the yield of ligation products. In some embodiments, a solution-phase splint fusion primer can be used in any of the nucleic acid amplification reactions of the present teachings, to generate amplicons of the target polynucleotide which include the universal sequence, where the amplicons can then bind to supports that bear only the universal primer sequence.

[00119] In some embodiments, a primer and/or target polynucleotide can include at least one universal sequence. A universal sequence is any nucleotide sequence that is present on many or substantially all of the primers or target polynucleotides. A universal sequence is a common sequence. For example, substantially all of the first polynucleotide-specific capture primers can include the same universal sequence. Optionally, the universal sequence can hybridize to an amplification primer or sequencing primer. Optionally, the universal sequence can include a sample-specific barcode sequence. Any of the target polynucleotides, capture primers or solution-phase primers can include at least one universal sequence. Optionally, substantially all of the supports within a first set of supports have a first universal sequence attached thereon, that is substantially identical among all supports within the first set of supports. Optionally, substantially all of the supports within a second set of supports have a second universal sequence attached thereon, that is substantially identical among all supports within the second set of supports. Optionally, substantially all of the supports within the first and second set of supports have a universal sequence attached thereon, that is substantially identical among all supports within the first and second set of supports.

[00120] In some embodiments, any oligonucleotide primer (e.g., capture, reverse solution-phase or fusion primer) can be compatible for use in any type of sequencing platform including chemical degradation, chain-termination, sequence-by-synthesis, pyrophosphate, massively parallel, ion-sensitive, and single molecule platforms. In some embodiments, any oligonucleotide primer can be compatible for use in any type of sequencing including: sequencing by oligonucleotide probe ligation and detection (e.g., SOLiD™), probe-anchor ligation sequencing (e.g., Complete Genomics or Polonator™), sequence-by-synthesis (e.g., Illumina), pyrophosphate sequencing (e.g., 454 Life Sciences), ion-sensitive sequencing (e.g., Personal Genome Machine (PGM™) and Ion Proton™ Sequencer, both from Ion Torrent Systems, Inc.) and single molecule sequencing platforms (e.g., Helicos™).

[00121] As used herein, a population of nucleic acids or polynucleotides is considered to be substantially “monoclonal” or is considered to have substantial “monoclonality” if a substantial portion of its members have substantially identical sequence. In some embodiments, members of a population need not be 100% identical, for example a certain number of “errors” may occur during the course of nucleic acid amplification reactions. In some embodiments, at least 50% of the members of a population are at least 90% identical to a reference nucleic acid molecule (i.e., a nucleic acid of defined sequence used as a basis for a sequence comparison). In some embodiments, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, or more of the members of a population comprise a substantially identical sequence compared to a reference nucleic acid molecule. In some embodiments, a low or insubstantial level of mixing of non-homologous nucleic acids may occur during nucleic acid amplification reactions described herein, and thus a clonal population may contain a minority of diverse nucleic acids (e.g., less than about 30%, less than about 20%, less than about 10%, less than about 5%, or less than about 1%, or less than about 0.1%).

[00122] For example, a nucleic acid amplification reaction can generate amplified polynucleotide strands having a sequence that is complementary to a template polynucleotide or having the same sequence as the template polynucleotide. A population of amplified polynucleotide strands is considered to be substantially monoclonal if a substantial portion of its members have a sequence that is substantially identical to a sequence that is complementary to the template sequence or have a sequence that is substantially identical to the template sequence.

[00123] In some embodiments, the disclosure relates generally to compositions, and related methods, systems, kits and apparatuses, comprising a single reaction mixture which can be used for a nucleic acid synthesis or nucleic acid amplification. The single reaction mixture can include any one or any combination of target polynucleotides, particles attached with capture primers, solution-phase primers, fusion primers, other additional primers, enzymes (e.g., polymerases), accessory proteins (e.g., recombinase, recombinase loading protein, single-stranded binding protein, helicase or topoisomerase), nucleotides, divalent cations, binding partners, co-factors and/or buffer. The single reaction mixture can include an inorganic phosphatase (e.g., from yeast), PEG and/or trehalose. The single reaction mixture can include betaine, trehalose, proline, Ficoll, PVP, PEG, dextran sulfate, DTT, DMSO, MMNO (4-methylmorpholine N-oxide) and/or PCR Boost™ (from Biomatrix, catalog No. 63301-011). In

some embodiments, the single reaction mixture contains an emulsion that provides compartmentalization for separately amplify target polynucleotides, or the single reaction mixture lacks an emulsion that provides compartmentalization. Optionally, the primers include any one or any combination of primers attached to a particle (e.g., immobilized capture primers) and/or soluble primers. Optionally, the enzymes comprise polymerases which include recombinant, fusion, mutant, heat-stable or heat labile forms. Optionally, the accessory proteins include any one or any combination of a single-stranded binding protein (e.g., SSB or gp32 protein), recombinase (e.g., recA or uvsX), recombinase loading protein (e.g., uvsY protein), helicase (e.g., uvsW protein), or topoisomerase. Optionally, the nucleotides can include compounds having structures the same as or similar to naturally-occurring nucleotides, or nucleotide analogs having derivatized base, sugar and/or phosphate groups, or labeled or non-labeled nucleotides. Optionally, the divalent cations include magnesium, manganese and/or calcium. Optionally, the binding partners include biotin and avidin-like compounds, such as avidin or streptavidin. Optionally, the buffer comprises a source of ions, such as KCl, K-acetate, NH₄-acetate, K-glutamate, NH₄Cl, or ammonium sulfate. Optionally, the buffer includes Tris, Tricine, HEPES, MOPS, ACES, MES, or inorganic buffers such as phosphate or acetate-based buffers which can provide a pH range of about 4-12. Optionally, the buffer includes chelating agents such as EDTA or EGTA. Optionally, the buffer includes dithiothreitol (DTT), glycerol, spermidine, and/or BSA (bovine serum albumin). Optionally, the buffer includes ATP.

[00124] In some embodiments, the nucleic acid amplification reaction is conducted in a single reaction mixture containing a single continuous liquid phase. As used herein, a “single continuous liquid phase” provides no substantial compartmentalization. For example, a single continuous liquid phase comprises only an aqueous phase. In some embodiments, a single continuous liquid phase lacks an oil phase. In some embodiments, a single continuous liquid phase lacks discrete aqueous phase droplets enclosed in an oil phase. In some embodiments, a single continuous liquid phase does not provide compartmentalization for multiple nucleic acid amplification reactions occurring in a single reaction vessel. In some embodiments, multiple nucleic acid amplification reactions occur in an aqueous phase in a single reaction vessel. In some embodiments, a single continuous liquid phase contains multiple nucleic acid amplification reactions that include multiple target polynucleotides (e.g., templates) having the same or different sequences.

[00125] In some embodiments, the nucleic acid amplification reaction is conducted in a single reaction mixture containing an emulsion comprising two immiscible liquid phases. In some embodiments, two immiscible liquid phases are mixed together to make the emulsion. In some embodiments, one of the liquid phases is dispersed in the other. In some embodiments, methods for nucleic acid amplification can be conducted in a discontinuous liquid phase. In some embodiments, methods for nucleic acid amplification can be conducted in a water-in-oil emulsion that provides compartmentalization (micro-reactors).

[00126] In some embodiments, the disclosure relates generally to compositions, and related methods, systems, kits and apparatuses, comprising a nucleic acid amplification reaction mixture distributed into one or more reaction vessels. In some embodiments, a single reaction vessel contains an amplification reaction mixture. Non-limiting examples of a single reaction vessel include a tube, inner wall of a tube, well, microwell, reaction chamber, groove, channel reservoir, flowcell, or similar structures.

[00127] In some embodiments, the nucleic acid amplification reaction mixture can be distributed directly into two or more reaction chambers arranged in an array. In some embodiments, the reaction chambers can be arranged in a grid or array. For example, an array can include two or more reaction chambers. Multiple reaction chambers can be arranged randomly or in an ordered array. An ordered array can include reaction chambers arranged in a single row, or in a two-dimensional grid with rows and columns. In some embodiments, the array can include one or more reaction chambers on a support. A reaction chamber can have walls and a bottom that define width and depth. The dimensions of a reaction chamber can be sufficient to permit deposition of reagents or for conducting nucleic acid amplification reactions. A reaction chamber can have any shape including cylindrical, polygonal or a combination of different shapes. Any wall of a reaction chamber can have a smooth or irregular surface. A reaction chamber can have a bottom with a planar, concave or convex surface. The bottom and side walls of a reaction chamber can comprise the same or different material and/or can be coated with a chemical group that can react with a biomolecule such as nucleic acids, proteins or enzymes.

[00128] An array can include any number of reaction chambers for depositing reagents and conducting numerous individual reactions. For example, an array can include at least 256 reaction chambers, or at least 256,000, or at least 1-3 million, or at least 3-5 million, or at least 5-

7 million, or at least 7-9 million, at least 9-11 million, at least 11-13 million reaction chambers, or even high density including 13-700 million reaction chambers or more. Reaction chambers arranged in a grid can have a center-to-center distance between adjacent reaction chambers (e.g., pitch) of less than about 10 microns, or less than about 5 microns, or less than about 1 microns, or less than about 0.5 microns.

[00129] An array can include reaction chambers having any width and depth dimensions. For example, a reaction chamber can have dimensions to accommodate a single microparticle (e.g., microbead) or multiple microparticles. A reaction chamber can hold about 0.001 – 100 picoliters of aqueous volume.

[00130] In some embodiments, at least one reaction chamber can be coupled to one or more sensors or can be fabricated above one or more sensors. A reaction chamber that is coupled to a sensor can provide confinement of reagents deposited therein so that products from a reaction can be detected by the sensor. A sensor can detect changes in products from any type of reaction, including any nucleic acid reaction such as primer extension, amplification or nucleotide incorporation reactions, within the reaction chamber. A sensor can detect changes (e.g., changes in the level or abundance) in ions (e.g., hydrogen ions), protons, phosphate groups such as pyrophosphate groups. A sensor can detect at least one by product of nucleotide incorporation, including pyrophosphate, hydrogen ions, charge transfer, or heat. In some embodiments, at least one reaction chamber can be coupled to one or more field effect transistor (FET), including for example an ion sensitive field effect transistor (ISFET). Examples of an array of reaction chambers coupled to ISFET sensors can be found at U.S. Patent No. 7,948,015, and U.S. serial No. 12/002,781, hereby incorporated by reference in their entireties. Other examples of sensors that detect byproducts of a nucleotide incorporation reaction can be found, for example, in Pourmand et al, Proc. Natl. Acad. Sci., 103: 6466-6470 (2006); Purushothaman et al., IEEE ISCAS, IV-169-172; Anderson et al, Sensors and Actuators B Chem., 129: 79-86 (2008); Sakata et al., Angew. Chem. 118:2283-2286 (2006); Esfandyapour et al., U.S. Patent Publication No. 2008/01666727; and Sakurai et al., Anal. Chem. 64: 1996-1997 (1992).

[00131] In some embodiments, a single reaction vessel can contain any one or any combination of reagents for conducting a nucleic acid amplification reaction, including a plurality of particles (e.g., individual particles in the plurality are attached with a plurality of a capture primer), a plurality of target polynucleotides, a plurality of solution-phase primers, one

or more polymerases, divalent cations and/or a plurality of nucleotides. In some embodiments, nucleic acid amplification reaction can further include any combination of a recombinase, recombinase accessory proteins, ATP, co-factors and/or one or more sieving agent or diffusion reducing agent. In some embodiments, any combination of reagents for conducting a nucleic acid amplification reaction can be deposited into a reaction vessel in any order, including sequentially or substantially simultaneously or a combination of both. In some embodiments, a nucleic acid amplification reaction can be conducted in a single reaction vessel comprising a single continuous liquid phase or an emulsion.

[00132] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising a plurality of target polynucleotides. In some embodiments, the target polynucleotides comprise single-stranded or double-stranded polynucleotides, or a mixture of both. In some embodiments, the target polynucleotides include polynucleotides having the same sequence or a mixture of different sequences. In some embodiments, the target polynucleotides include polynucleotides having the same or different lengths. In some embodiments, the plurality of target polynucleotides includes about 2-10, or about 10-50, or about 50-100, or about 100-500, or about 500-1,000, or about 1,000 – 5,000, or about $10^3 - 10^6$, or about $10^6 - 10^{10}$ or more target polynucleotide molecules. In some embodiments, the target polynucleotides comprise polymers of deoxyribonucleotides, ribonucleotides, and/or analogs thereof. In some embodiments, the target polynucleotides comprise naturally-occurring, synthetic, recombinant, cloned, fragmented, amplified, unamplified or archived (e.g., preserved) forms. In some embodiments, the target polynucleotides comprise DNA, cDNA, RNA, RNA/DNA, or nucleic acid analogs. In some embodiments, the target polynucleotides comprise mRNA, miRNA, rRNA or tRNA.

[00133] In some embodiments, the target polynucleotides lack any nucleic acid adaptor. For example, the target polynucleotides are not joined or appended to a nucleic acid adaptor. In some embodiments, at least a portion of the target polynucleotide can hybridize to the capture primer, fusion primer, solution-phase primer, amplification primer or sequencing primers.

[00134] In some embodiments, the target polynucleotides have one or both ends joined to a nucleic acid adaptor. For example, the first end of a target polynucleotide can be joined to a first nucleic acid adaptor. Optionally, the second end of the target polynucleotide can be joined to a second nucleic acid adaptor. The first and second adaptors can have the same or different

sequence. In some embodiments, at least a portion of the first or second nucleic acid adaptor can hybridize to the capture primer, fusion primer, solution-phase primer, amplification primer or sequencing primers.

[00135] In some embodiments, target polynucleotides can be compatible for use in any type of sequencing platform including chemical degradation, chain-termination, sequence-by-synthesis, pyrophosphate, massively parallel, ion-sensitive, and single molecule sequencing platforms.

[00136] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising one or more nucleic acid samples containing polynucleotides, including target polynucleotides (e.g., polynucleotides-of-interest) and/or non-target polynucleotides. In some embodiments, the nucleic acid samples can be isolated from a biological sample, including a biological fluid, cell culture, solid tissue or each nucleic acid sample can be isolated from a single cell. In some embodiments, the nucleic acid sample comprises genomic DNA. In some embodiments, nucleic acid samples can originate from any organism including human, canine, feline, bovine, equine, murine, porcine, caprine, lupine, ranine, piscine, simian, ape, plant, insect, bacteria, virus or fungus. In some embodiments, the nucleic acid sample can originate from water, soil or food. In some embodiments, the nucleic acid sample can be a biological sample, which includes a biological fluid obtained from blood, serum, plasma, saliva, sputum, sweat, tears, lavage fluid, amniotic fluid, cerebrospinal fluid, ascites, urine, semen and the like. For example, blood, serum and plasma include fractions or processed portions thereof. Optionally, the target polynucleotide can be extracted from a formalin fixed paraffin-embedded (FFPE) sample. In some embodiments, a biological sample includes a biological fluid or solid tissue obtained by biopsy, swab, or smear. In some embodiments, the solid tissue includes healthy or diseased tissue (e.g., tumor) or fluid, or a mixture of healthy and diseased tissue or fluid. In some embodiments, the nucleic acid sample originate from a biological sample that contains cells, bacteria, virus, fungus and/or cell-free nucleic acids.

[00137] In some embodiments, the nucleic acid sample can undergo a separate processing step to extract the polynucleotides, and the extracted polynucleotides can be used to conduct any of the amplification methods of the present teachings. In some embodiments, an optional enrichment step can be performed to remove the cellular debris. For example, cells (or a single cell) contained within a biological fluid can be lysed to release the polynucleotides which are

then enriched or purified to remove the cellular debris. In some embodiments, the nucleic acid sample can be used directly in a tag-appending reaction without any separate polynucleotide extraction step. For example, the nucleic acid sample (e.g., blood) is added directly to a reaction vessel along with various reagents for conducting a nucleic acid amplification reaction. That is, the cells (or the single cell) contained within the biological fluid are lysed in the nucleic acid amplification reaction mixture to release the target polynucleotides. In some embodiments, a separate cell lysis step is not practiced, or a lysis step is conducted prior to the nucleic acid amplification step. In some embodiments, the target polynucleotide is from cell-free DNA or RNA contained within the biological fluid. In some embodiments, a biological sample includes a biological fluid or solid tissue obtained by biopsy, swab, or smear. In some embodiments, the solid tissue includes healthy or diseased tissue, or a mixture of both.

[00138] In some embodiments, the nucleic acid sample is obtained from a healthy subject, or from a subject having a disease, or from a subject suspected of having a disease. In some embodiments, the nucleic acid sample is obtained from a subject having a disease or from a subject suspected of having a disease. Optionally, the disease is a genetic disease or an infectious disease. In some embodiments, the disease is an infectious disease caused by an infectious agent selected from a bacterium, a mycobacterium, a virus, a fungus, and a parasite. In some embodiments, the disease is sepsis, pneumonia, MRSA, genitourinary tract infection, tuberculosis, hepatitis, HIV, candidiasis, or malaria. In some embodiments, the disease includes water-borne or food-borne diseases from water or food contaminated with one or more pathogens. In some embodiments, the pathogen itself causes the disease or toxins produced by the pathogen cause the disease. In some embodiments, the disease can be caused by *Campylobacter*, *Salmonella*, *Shigella*, *E. coli*, *Listeria*, *Staphylococcus*, *Clostridium*, and/or novovirus.

[00139] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising target polynucleotides and at least one adaptor.

[00140] In some embodiments, the target polynucleotides are joined or appended to at least one adaptor. In some embodiments, the target polynucleotides lack any adaptor. In some embodiments, one or more adaptors can be joined to the target polynucleotide by ligation. In some embodiments, a tailed amplification primer can be used in a PCR reaction to append one or

more adaptors to a target polynucleotide, where the tailed amplification primer includes the sequence of one or more adaptors.

[00141] In some embodiments, the adaptor comprises a nucleic acid, including DNA, RNA, RNA/DNA molecules, or analogs thereof. In some embodiments, the adaptor can include one or more deoxyribonucleoside or ribonucleoside residues. In some embodiments, the adaptor can be single-stranded or double-stranded nucleic acids, or can include single-stranded and/or double-stranded portions. In some embodiments, the adaptor can have any structure, including linear, hairpin, forked (Y-shaped), or stem-loop.

[00142] In some embodiments, the adaptor can have any length, including fewer than 10 bases in length, or about 10-20 bases in length, or about 20-50 bases in length, or about 50-100 bases in length, or longer.

[00143] In some embodiments, the adaptor can have any combination of blunt end(s) and/or sticky end(s). In some embodiments, at least one end of the adaptor can be compatible with at least one end of a nucleic acid fragment. In some embodiments, a compatible end of the adaptor can be joined to a compatible end of a nucleic acid fragment. In some embodiments, the adaptor can have a 5' or 3' overhang end. In some embodiments, the adaptor can have a 5' or 3' overhang tail. In some embodiments, the tail can be any length, including 1-50 or more nucleotides in length.

[00144] In some embodiments, the adaptor can include an internal nick. In some embodiments, the adaptor can have at least one strand that lacks a terminal 5' phosphate residue. In some embodiments, the adaptor lacking a terminal 5' phosphate residue can be joined to a nucleic acid fragment to introduce a nick at the junction between the adaptor and the nucleic acid fragment.

[00145] In some embodiments, the adaptor can include a nucleotide sequence that is identical or complementary to any portion of the target polynucleotide, capture primer, fusion primer, solution-phase primer, amplification primer, or a sequencing primer.

[00146] In some embodiments, the adaptor can include a unique identifier sequence (e.g., sample-specific barcode sequence). In some embodiments, a barcoded adaptor can be used for constructing a multiplex library of target polynucleotides. In some embodiments, the barcoded adaptors can be appended to a target polynucleotide and used for sorting or tracking the source of the target polynucleotide. In some embodiments, one or more barcode sequences can allow

identification of a particular adaptor among a mixture of different adaptors having different barcodes sequences. For example, a mixture can include 2, 3, 4, 5, 6, 7-10, 10-50, 50-100, 100-200, 200-500, 500-1000, or more different adaptors having unique barcode sequences.

[00147] In some embodiments, the adaptor can include degenerate sequences. In some embodiments, the adaptor can include one or more inosine residues.

[00148] In some embodiments, the adaptor can include at least one scissile linkage. In some embodiments, the scissile linkage can be susceptible to cleavage or degradation by an enzyme or chemical compound. Optionally, the adaptor includes at least one uracil base. In some embodiments, the adaptor can include at least one phosphorothiolate, phosphorothioate, and/or phosphoramidate linkage.

[00149] In some embodiments, the adaptor can include any type of restriction enzyme recognition sequence, including type I, type II, type IIs, type IIB, type III, type IV restriction enzyme recognition sequences, or recognition sequences having palindromic or non-palindromic recognition sequences.

[00150] In some embodiments, the adaptor can include a cell regulation sequences, including a promoter (inducible or constitutive), enhancers, transcription or translation initiation sequence, transcription or translation termination sequence, secretion signals, Kozak sequence, cellular protein binding sequence, and the like.

[00151] In some embodiments, a target polynucleotide does not comprise an adaptor. In some embodiments, a target polynucleotide does not comprise a universal adaptor. A universal adaptor can be a sequence suitable for use as a primer hybridization site that is joined or appended to multiple target polynucleotides in a sample, such as the majority of or substantially all of the multiple target nucleotides in a sample. In some embodiments, the multiple target polynucleotides not having identical sequences.

[00152] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising one or more polymerases. In some embodiments, the compositions (and related methods, systems, kits and apparatuses) includes one type, or a mixture of different types of polymerases. In some embodiments, the polymerase includes any enzyme, or fragment or subunit of thereof, that can catalyze polymerization of nucleotides and/or nucleotide analogs. In some embodiments, the polymerase requires a nucleic

acid having an extendible 3' end. For example, the polymerase can require a terminal 3' OH of a nucleic acid primer to initiate nucleotide polymerization.

[00153] The polymerase comprises any enzyme that can catalyze the polymerization of nucleotides (including analogs thereof) into a nucleic acid strand. Typically but not necessarily such nucleotide polymerization can occur in a template-dependent fashion. In some embodiments, the polymerase can be a high fidelity polymerase. Such polymerases can include without limitation naturally occurring polymerases and any subunits and truncations thereof, mutant polymerases, variant polymerases, recombinant, fusion or otherwise engineered polymerases, chemically modified polymerases, synthetic molecules or assemblies, and any analogs, derivatives or fragments thereof that retain the ability to catalyze such polymerization. Optionally, the polymerase can be a mutant polymerase comprising one or more mutations involving the replacement of one or more amino acids with other amino acids, the insertion or deletion of one or more amino acids from the polymerase, or the linkage of parts of two or more polymerases. The term "polymerase" and its variants, as used herein, also refers to fusion proteins comprising at least two portions linked to each other, where the first portion comprises a peptide that can catalyze the polymerization of nucleotides into a nucleic acid strand and is linked to a second portion that comprises a second polypeptide, such as, for example, a reporter enzyme or a processivity-enhancing domain. Typically, the polymerase comprises one or more active sites at which nucleotide binding and/or catalysis of nucleotide polymerization can occur. In some embodiments, the polymerase includes or lacks other enzymatic activities, such as for example, 3' to 5' exonuclease activity or 5' to 3' exonuclease activity. In some embodiments, the polymerase can be isolated from a cell, or generated using recombinant DNA technology or chemical synthesis methods. In some embodiments, the polymerase can be expressed in prokaryote, eukaryote, viral, or phage organisms. In some embodiments, the polymerase can be post-translationally modified proteins or fragments thereof.

[00154] In some embodiments, the polymerase can be a DNA polymerase and include without limitation bacterial DNA polymerases, eukaryotic DNA polymerases, archaeal DNA polymerases, viral DNA polymerases and phage DNA polymerases. In some embodiments, the polymerase can be a Bst polymerase from *Bacillus stearothermophilus*, a Bsu polymerase from *Bacillus subtilis*, or a Sau polymerase from *Staphylococcus aureus*.

[00155] In some embodiments, the polymerase can be a replicase, DNA-dependent polymerase, primases, RNA-dependent polymerase (including RNA-dependent DNA polymerases such as, for example, reverse transcriptases), a thermo-labile polymerase, or a thermo-stable polymerase. In some embodiments, the polymerase can be any Family A or B type polymerase. Many types of Family A (e.g., *E. coli* Pol I), B (e.g., *E. coli* Pol II), C (e.g., *E. coli* Pol III), D (e.g., Euryarchaeotic Pol II), X (e.g., human Pol beta), and Y (e.g., *E. coli* UmuC/DinB and eukaryotic RAD30/xeroderma pigmentosum variants) polymerases are described in Rothwell and Watsman 2005 *Advances in Protein Chemistry* 71:401-440. In some embodiments, a polymerase can be a T3, T5, T7, or SP6 RNA polymerase. The T7 DNA polymerase can be used with thioredoxin.

[00156] In some embodiments, the polymerase comprises a heat-stable polymerase. In some embodiments, the polymerase comprises a heat-labile polymerase. In some embodiments, the polymerase comprises a low fidelity polymerase. In some embodiments, the polymerase comprises a high fidelity polymerase.

[00157] In some embodiments, the polymerase can lack 5' – 3' exonuclease activity. In some embodiments, the polymerase can have strand-displacement activity.

[00158] In some embodiments, the archaeal DNA polymerase, can be, without limitation, a thermostable or thermophilic DNA polymerase such as, for example: a *Bacillus subtilis* (Bsu) DNA polymerase I large fragment; a *Thermus aquaticus* (Taq) DNA polymerase; a *Thermus filiformis* (Tfi) DNA polymerase; a Phi29 DNA polymerase; a *Bacillus stearothermophilus* (Bst) DNA polymerase; a *Thermococcus sp.* 9° N-7 DNA polymerase; a *Bacillus smithii* (Bsm) DNA polymerase large fragment; a *Thermococcus litoralis* (Tli) DNA polymerase or VentTM (exo-) DNA polymerase (from New England Biolabs); or “Deep Vent” (exo-) DNA polymerase (New England Biolabs). In some embodiments, the polymerase comprises *E. coli* large fragment DNA polymerase I (e.g., Klenow).

[00159] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising at least one accessory protein. In some embodiments, the accessory protein can bind single-stranded or double-stranded nucleic acids. Optionally, the accessory protein can mediate loading other proteins (e.g., recombinase) onto a nucleic acid. Optionally, the accessory protein can unwind nucleic acid substrates, relax nucleic acids, resolve nucleic acid structures, hydrolyze nucleic acids (e.g., nuclease),

disassemble complexes of nucleic acids and proteins, or disassemble nucleic acid structures. Optionally, the accessory protein can partially or fully denature a double-stranded first or second target nucleic acid. Optionally, the accessory protein can catalyze strand invasion or unwinding. Optionally, the accessory protein comprises a sliding clamp protein. Optionally, the accessory protein can mediate or catalyze its respective activity in a sequence-specific or sequence-independent manner.

[00160] In some embodiments, the accessory protein comprises wild-type, mutant, recombinant, fusion, or fragments thereof. In some embodiments, an accessory protein comprises a multimeric protein complex. Optionally, the multimeric protein complex comprises 2, 3, 4, 5, 6, 7, 8, or more subunits. Optionally, the multimeric accessory protein complex comprises a homo-meric or hetero-meric protein complex.

[00161] In some embodiments, the accessory proteins can originate from any bacteriophage including a myoviral phage. The accessory proteins can originate from bacteriophage T2, T4, T5 or T7. The accessory proteins can originate from any prokaryote, archaeon, bacterium (e.g., *E. coli*), eukaryote, or mammal (e.g., human).

[00162] In some embodiments, the accessory proteins comprise a single-stranded binding protein including myoviral gp32 (e.g., T4 or RB69), Sso SSB from *Sulfolobus solfataricus*, MjA SSB from *Methanococcus jannaschii*, or *E. coli* SSB protein.

[00163] In some embodiments, the single reaction mixture comprises a mixture of different accessory proteins that originate from the same or different species. Optionally, the single reaction mixture comprises a mixture of different accessory proteins that originate from the same or different species as a recombinase enzyme.

[00164] In some embodiment, the accessory protein comprises a single-stranded binding protein (e.g., SSB or gp32), recombinase (e.g., recA or uvsX), recombinase loading protein (e.g., uvsY), helicase (e.g., uvsW), or topoisomerase.

[00165] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising at least one enzyme that catalyzes homologous recombination. For example an enzyme that catalyzes homologous recombination can bind an oligonucleotide primer (e.g., capture primer, reverse solution-phase primer or fusion primer) to form a nucleoprotein complex. In some embodiments, a homologous recombination enzyme can catalyze strand invasion by forming a nucleoprotein complex and binding to a

homologous portion of a double-stranded polynucleotide to form a recombination intermediate having a triple-strand structure (e.g., D-loop formation).

[00166] In some embodiments, the enzyme that catalyzes homologous recombination comprises wild-type, mutant, recombinant, fusion, or fragments thereof. In some embodiments, a homologous recombination enzyme comprises at least a portion of a recombinase enzyme from any organism, including bacteriophages (e.g., *uvrX*, such as bacteriophage T4 *uvrX*), bacteria (e.g., *recA*, such as *Escherichia coli recA*), or eukaryotes (e.g., RAD51, such as human or *Saccharomyces cerevisiae* RAD51).

[00167] In some embodiments, the enzyme that catalyzes homologous recombination comprises a recombinase enzyme, such as a member of the *uvrX/recA/RAD51* family.

[00168] In some embodiments, the recombinase can form a nucleoprotein complex by binding a capture primer. Optionally, the nucleoprotein complex further includes a target polynucleotide, where a portion of the capture primer hybridizes to a portion of the target polynucleotide. Optionally, the target polynucleotide comprises a double-stranded polynucleotide molecule. Optionally, the recombinase can partially or fully denature the double-stranded first target nucleic acid.

[00169] In some embodiments, the recombinase can form a nucleoprotein complex by binding a solution-phase primer. Optionally, the nucleoprotein complex further includes a target polynucleotide, where a portion of the solution-phase primer hybridizes to a portion of the target polynucleotide. Optionally, the target polynucleotide comprises a double-stranded polynucleotide molecule. Optionally, the recombinase can partially or fully denature the double-stranded second target nucleic acid.

[00170] In some embodiments, the recombination enzyme comprises at least a portion of a recombinase enzyme from any organism, including *Escherichia coli* (e.g., *recA*), human (e.g., RAD51), or bacteriophage T4 (e.g., *uvrX*) (U.S. patent No. 5,223,414 to Zarling, U.S. patent Nos. 5,273,881 and 5,670,316 both to Sena, and U.S. Patent Nos. 7,270,981, 7,399,590, 7,435,561, 7,666,598, 7,763,427, 8,017,339, 8,030,000, 8,062,850, and 8,071,308).

[00171] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising at least one accessory protein that improves the activity of a recombinase enzyme (see, e.g., U.S. patent 8,071,308 granted to Piepenburg, et al.). In some embodiments, an accessory protein can bind single strands of

nucleic acids, or can load a recombinase onto a nucleic acid. In some embodiments, an accessory protein comprises wild-type, mutant, recombinant, fusion, or fragments thereof. In some embodiments, an accessory protein and a recombinase enzyme can originate from any combination of the same or different species. Accessory proteins can originate from any bacteriophage including a myoviridae phage. Examples of a myoviridae phage include T4, T2, T6, Rb69, Aeh1, KVP40, *Acinetobacter* phage 133, *Aeromonas* phage 65, cyanophage P-SSM2, cyanophage PSSM4, cyanophage S-PM2, Rb14, Rb32, *Aeromonas* phage 25, *Vibrio* phage nt-1, phi-1, Rb16, Rb43, Phage 31, phage 44RR2.8t, Rb49, phage Rb3, and phage LZ2 (U.S. patent 8,071,308 granted to Piepenburg). Accessory proteins can originate from any bacterial species, including *Escherichia coli*, *Sulfolobus* (e.g., *S. solfataricus*) or *Methanococcus* (e.g., *M. jannaschii*).

[00172] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising at least one co-factor for polymerase or recombinase activity. In some embodiments, a co-factor comprises one or more divalent cation. Examples of divalent cations include magnesium, manganese and calcium.

[00173] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising at least one co-factor for polymerase or recombinase assembly on nucleic acids or for homologous nucleic acid pairing. In some embodiments, a co-factor comprise any form of ATP including ATP and ATP γ S.

[00174] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising at least one co-factor that regenerates ATP. For example, a co-factor comprises an enzyme system that converts ADP to ATP. In some embodiments, a co-factor comprises phosphocreatine and/or creatine kinase.

[00175] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising one or more nucleotides. In some embodiments, the compositions (and related methods, systems, kits and apparatuses) includes one type, or a mixture of different types of nucleotides. A nucleotide comprises any compound that can bind selectively to, or can be polymerized by, a polymerase. Typically, but not necessarily, selective binding of the nucleotide to the polymerase is followed by polymerization of the nucleotide into a nucleic acid strand by the polymerase. Such nucleotides include not only naturally occurring nucleotides but also any analogs, regardless of their structure, that can bind

selectively to, or can be polymerized by, a polymerase. While naturally occurring nucleotides typically comprise base, sugar and phosphate moieties, the nucleotides of the present disclosure can include compounds lacking any one, some or all of such moieties. In some embodiments, the nucleotide can optionally include a chain of phosphorus atoms comprising three, four, five, six, seven, eight, nine, ten or more phosphorus atoms. In some embodiments, the phosphorus chain can be attached to any carbon of a sugar ring, such as the 5' carbon. The phosphorus chain can be linked to the sugar with an intervening O or S. In some embodiments, one or more phosphorus atoms in the chain can be part of a phosphate group having P and O. In some embodiments, the phosphorus atoms in the chain can be linked together with intervening O, NH, S, methylene, substituted methylene, ethylene, substituted ethylene, CNH₂, C(O), C(CH₂), CH₂CH₂, or C(OH)CH₂R (where R can be a 4-pyridine or 1-imidazole). In some embodiments, the phosphorus atoms in the chain can have side groups having O, BH₃, or S. In the phosphorus chain, a phosphorus atom with a side group other than O can be a substituted phosphate group. In the phosphorus chain, phosphorus atoms with an intervening atom other than O can be a substituted phosphate group. Some examples of nucleotide analogs are described in Xu, U.S. Patent No. 7,405,281.

[00176] Some examples of nucleotides that can be used in the disclosed compositions (and related methods, systems, kits and apparatuses) include, but are not limited to, ribonucleotides, deoxyribonucleotides, modified ribonucleotides, modified deoxyribonucleotides, ribonucleotide polyphosphates, deoxyribonucleotide polyphosphates, modified ribonucleotide polyphosphates, modified deoxyribonucleotide polyphosphates, peptide nucleotides, modified peptide nucleotides, metallonucleosides, phosphonate nucleosides, and modified phosphate-sugar backbone nucleotides, analogs, derivatives, or variants of the foregoing compounds, and the like. In some embodiments, the nucleotide can comprise non-oxygen moieties such as, for example, thio- or borano- moieties, in place of the oxygen moiety bridging the alpha phosphate and the sugar of the nucleotide, or the alpha and beta phosphates of the nucleotide, or the beta and gamma phosphates of the nucleotide, or between any other two phosphates of the nucleotide, or any combination thereof. In some embodiments, a nucleotide can include a purine or pyrimidine base, including adenine, guanine, cytosine, thymine, uracil or inosine. In some embodiments, a nucleotide includes dATP, dGTP, dCTP, dTTP and dUTP.

[00177] In some embodiments, the nucleotide is unlabeled. In some embodiments, the nucleotide comprises a label and referred to herein as a “labeled nucleotide”. In some embodiments, the label can be in the form of a fluorescent dye attached to any portion of a nucleotide including a base, sugar or any intervening phosphate group or a terminal phosphate group, i.e., the phosphate group most distal from the sugar.

[00178] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising any one or any combination of capture primers, reverse solution-phase primers, fusion primers, target polynucleotides and/or nucleotides that are non-labeled or attached to at least one label. In some embodiments, the label comprises a detectable moiety. In some embodiments, the label can generate, or cause to generate, a detectable signal. In some embodiments, the detectable signal can be generated from a chemical or physical change (e.g., heat, light, electrical, pH, salt concentration, enzymatic activity, or proximity events). For example, a proximity event can include two reporter moieties approaching each other, or associating with each other, or binding each other. In some embodiments, the detectable signal can be detected optically, electrically, chemically, enzymatically, thermally, or via mass spectroscopy or Raman spectroscopy. In some embodiments, the label can include compounds that are luminescent, photoluminescent, electroluminescent, bioluminescent, chemiluminescent, fluorescent, phosphorescent or electrochemical. In some embodiments, the label can include compounds that are fluorophores, chromophores, radioisotopes, haptens, affinity tags, atoms or enzymes. In some embodiments, the label comprises a moiety not typically present in naturally occurring nucleotides. For example, the label can include fluorescent, luminescent or radioactive moieties.

[00179] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising at least one member of a binding partner. In some embodiments, a binding partners includes two molecules, or portions thereof, which have a specific binding affinity for one another and typically will bind to each other in preference to binding to other molecules. In some embodiments, binding partners include an “affinity moiety” and a “receptor moiety”. Typically but not necessarily some or all of the structure of one member of a specific binding pair is complementary to some or all of the structure possessed by the other member, with the two members being able to bind together

specifically by way of a bond between the complementary structures, optionally by virtue of multiple non-covalent attractions.

[00180] In some embodiments, molecules that function as binding partners include: biotin (and its derivatives) and its binding partners avidin, streptavidin and their derivatives; His-tags which bind nickel, cobalt or copper; cysteine, histidine, or histidine patch which bind Ni-NTA; maltose which binds with maltose binding protein (MBP); lectin-carbohydrate binding partners; calcium-calcium binding protein (CBP); acetylcholine and receptor-acetylcholine; protein A and binding partner anti-FLAG antibody; GST and binding partner glutathione; uracil DNA glycosylase (UDG) and ugi (uracil-DNA glycosylase inhibitor) protein; antigen or epitope tags which bind to antibody or antibody fragments, particularly antigens such as digoxigenin, fluorescein, dinitrophenol or bromodeoxyuridine and their respective antibodies; mouse immunoglobulin and goat anti-mouse immunoglobulin; IgG bound and protein A; receptor-receptor agonist or receptor antagonist; enzyme-enzyme cofactors; enzyme-enzyme inhibitors; and thyroxine-cortisol. Another binding partner for biotin can be a biotin-binding protein from chicken (Hytonen, et al., BMC Structural Biology 7:8).

[00181] In some embodiments, an avidin moiety can include an avidin protein, as well as any derivatives, analogs and other non-native forms of avidin that can bind to biotin moieties. Other forms of avidin moieties include native and recombinant avidin and streptavidin as well as derivatized molecules, e.g. nonglycosylated avidins, N-acyl avidins and truncated streptavidins. For example, avidin moiety includes deglycosylated forms of avidin, bacterial streptavidins produced by *Streptomyces* (e.g., *Streptomyces avidinii*), truncated streptavidins, recombinant avidin and streptavidin as well as to derivatives of native, deglycosylated and recombinant avidin and of native, recombinant and truncated streptavidin, for example, N-acyl avidins, e.g., N-acetyl, N-phthalyl and N-succinyl avidin, and the commercial products ExtrAvidinTM, CaptavidinTM, NeutravidinTM and Neutralite AvidinTM.

[00182] When conducting a nucleic acid amplification reaction with a plurality of target polynucleotides and particles in a single reaction vessel, the target polynucleotides will tend to move randomly from one particle to another due to Brownian motion. Polynucleotide migration increases the incidence of polyclonal amplification at each particle. One technique to reduce polyclonality employs physical barriers to separate one polynucleotide and one particle from other polynucleotides and particles. For example, a water-in-oil emulsion can form aqueous

microreactors that provide physical barriers (e.g., compartments) for performing many separate amplification reactions in a single reaction vessel. In some embodiments, Brownian motion in a nucleic acid amplification reaction can be reduced by adding a sieving agent or a diffusion-reducing agent to a single continuous liquid phase. In some embodiments, improving monoclonality of the amplified polynucleotides attached to a particle comprises adding a sieving agent and/or a diffusion-reducing agent to a single continuous liquid phase. In some embodiments, methods for nucleic acid amplification can be conducted with a sieving agent or a diffusion-reducing agent. In some embodiments, a sieving agent or a diffusion-reducing agent can reduce migration of the polynucleotide away from a support (e.g., a particle or bead) during the amplification reaction.

[00183] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising a single reaction mixture that includes at least one sieving agent. For example, a nucleic acid amplification reaction mixture can include at least one sieving agent. In some embodiments, a sieving agent comprises any compound that can provide a physical barrier. In some embodiments, a sieving agent provides a molecular sieve. In some embodiments, a sieving agent comprises any compound that can provide a matrix having a plurality of pores that are small enough to reduce the movement/migration of any component of a nucleic acid amplification reaction. For example, components of a nucleic acid amplification reaction include any one or any combination of particles attached with a plurality of capture primers, target polynucleotides, recombinase, polymerase, solution-phase primers, fusion primers, nucleotides, divalent cations, ATP and/or co-factors. For example, a sieving agent can reduce the movement of a target polynucleotide (or a polynucleotide associated with a surface or particle) through the pores. In some embodiments, a sieving agent comprises any compound that can provide a matrix having a plurality of pores that are small enough to slow the movement of a target polynucleotide away from a surface (e.g., particle or planar surface). Thus, a sieving agent can reduce Brownian motion of a target polynucleotide or any other component of a nucleic acid amplification reaction. In some embodiments, a sieving agent can be selected to form pore sizes small enough to reduce movement of a target polynucleotide through the pores, but large enough to permit movement of smaller components in a nucleic acid amplification reaction, such as cations, nucleotides, divalent cations, ATP and co-factors. In some embodiments, the pore size or range of pore sizes

can be modulated by increasing or decreasing the concentration of a sieving agent. For example, the molecular weight, intrinsic viscosity and concentration of a sieving agent (or a combination of sieving agents) can be selected to prepare a nucleic acid amplification reaction mixture in a particular solvent (e.g., water) to produce a matrix having a desired pore size or viscosity. In some embodiments, a sieving agent can reduce bulk flow by increasing the viscosity of a nucleic acid amplification reaction mixture. In some embodiments, a sieving agent can be water soluble. In some embodiments, a matrix having a plurality of pores can be prepared by mixing a sieving agent with a solvent (e.g., an aqueous solvent, such as water). In some embodiments, a sieving agent does not interfere with nucleic acid amplification or formation of a recombinase nucleoprotein complex. In some embodiments, conducting a nucleic acid amplification reaction with one or more sieving agents can reduce the movement of a polynucleotide away from a particular particle and can increase the likelihood that the polynucleotide will hybridize with a capture primer attached to a particular particle, where the primer provides an initiation site for nucleotide polymerization which can increase monoclonality of the polynucleotides attached to the particular particle. In some embodiments, the percentage of particle that are attached with a monoclonal population of a polynucleotide can be increased by conducting a nucleic acid amplification reaction with at least one sieving agent.

[00184] In some embodiments, a sieving agent comprises a polymer compound. In some embodiments, a sieving agent comprises cross-linked or non-cross linked forms. In some embodiments, a sieving agent comprises linear or branched forms. In some embodiments, a sieving agent comprises charged or neutral forms. In some embodiments, a polymer sieving agent comprises an average molecular weight of about 10,000 – 2,000,000, or about 12,000-95,000, or about 13,000-100,000. In some embodiments, a sieving agent comprises a viscosity range of about 5 centipoise to about 15,000 centipoise when dissolved in water at 2 weight percent measured at about 25°C, or about 10 centipoise to about 10,000 centipoise as a 2% aqueous solutions measured at about 25°C, or about 15 centipoise to about 5,000 centipoise as a 2% aqueous solution measured at about 25°C. In some embodiments, a sieving agent comprises a viscosity average molecular weight of about 25 to about 1,500 kMv, or about 75-1,000 kMv, or about 85-800 kMv. In some embodiments, a nucleic acid amplification reaction mixture comprises a polymer at about 0.1 to about 20% weight per volume, or about 1-10% w/v, or about 2-5% w/v. For example, a sieving agent comprises an acrylamide polymer including

polyacrylamide. In some embodiments, a sieving agent comprises a saccharide polymer. In some embodiments, a sieving agent comprises a polymer of glucose or galactose. In some embodiments, a saccharide polymers comprises cellulose, dextran, starch, glycogen, agar or agarose. In some embodiments, a sieving agent comprises sodium carboxymethyl 2-hydroxyethyl cellulose, sodium carboxymethyl cellulose, methyl cellulose, hydroxyl ethyl cellulose, 2-hydroxypropyl cellulose, carboxy methyl cellulose, hydroxyl propyl cellulose, hydroxyethyl methyl cellulose, hydroxybutyl methyl cellulose, (hydroxypropyl)methyl cellulose or hydroxyethyl ethyl cellulose. In some embodiments, a nucleic acid amplification reaction mixture comprises a mixture of different sieving agents, for example, a mixture of different cellulose derivatives.

[00185] In some embodiments, the present teachings provide methods for nucleic acid amplification, comprising at least one diffusion-reducing agent. In some embodiments, a diffusion-reducing agent comprises any compound that reduces diffusion/migration of polynucleotides from a region of higher concentration to one having a lower concentration. In some embodiments, a diffusion reducing agent comprises any compound that reduces migration of any component of a nucleic acid amplification reaction irrespective of size. In some embodiments, components of a nucleic acid amplification reaction include any one or any combination of particles attached with capture primers, polynucleotides, recombinase, polymerase, solution-phase primers, fusion primers nucleotides, divalent cations, ATP and/or co-factors. In some embodiments, a diffusion reducing agent comprises any compound that behaves as a crowding agent (U.S. patent 8,071,308 granted to Piepenburg). For example, a crowding agent can increase the concentration of one or more components in a nucleic acid amplification reaction by generating a crowded reaction environment. In some embodiments, a diffusion reducing agent comprises oligomers or polymers of ethylene oxide including polyethylene glycol (PEG), polyethylene oxides including triblock copolymers (e.g., PluronicTM), polystyrene, Ficoll, dextran, polyvinylpyrrolidone (PVP), or albumin.

[00186] In some embodiments, the nucleic acid amplification reaction includes a polymerase chain reaction (PCR) (U.S. patent 4,683,195 and 4,683,202 both granted to Mullis), ligase chain reaction (LCR) (Barany 1991 Proceedings National Academy of Science USA 88:189-193; Barnes 1994 Proceedings National Academy of Science USA91:2216-2220), or isothermal self-sustained sequence reaction (Kwoh 1989 Proceedings National Academy of Science USA

86:1173-1177; WO 1988/10315; and U.S. patents 5,409,818, 5,399,491, and 5,194,370), or recombinase polymerase amplification (RPA) (U.S. patent No. 5,223,414 to Zarling; U.S. patent Nos. 5,273,881 and 5,670,316 both to Sena; U.S. patent Nos. 7,270,981, 7,399,590, 7,435,561, 7,666,598, 7,763,427, 8,017,339, 8,030,000, 8,062,850, and 8,071,308; and U.S. patent Nos. 9,309,557, 9,309,558, 9,334,531 and 9,371,557 issued to Li, et al.).

[00187] PCR is a DNA synthesis reaction in which the reaction mixture is subjected to at least two complete reaction cycles, each reaction cycle comprising a denaturation period and at least one annealing and/or extension period, resulting in synthesis of copies of a nucleic acid template in at least the initial cycles, and copies of the copies in at least the later cycles, generally resulting in geometric amplification of the template. In PCR, a pair of primers are provided that bind at each end of a target region, on opposite strands such that they each prime synthesis toward the other primer. The reaction is thermo-cycled so as to drive denaturation of the substrate in a high temperature step, annealing of the primers at a lower temperature step, and extension at a temperature which may be but is not necessarily higher than that of the annealing step. Geometric amplification occurs because the products of one cycle can serve as template in the next cycle.

[00188] LCR is a reaction in which at least a first probe and a second probe are provided. In an embodiment of LCR, the first and second probes are ligated in the presence of a template. The probes generally hybridize to the template to form a substrate for a ligase, i.e., a structure resembling nicked double-stranded DNA. A DNA ligase, such as a thermostable DNA ligase, is provided and product (ligated probes) is generated in a cyclic manner. A cycle of the reaction involves a denaturation step, an annealing step, and a ligation step. The reaction can provide geometric amplification, e.g., where probes are provided that hybridize to both strands of a double-stranded template, or where third and fourth probes complementary to the first and second probes are provided, such that the product from one cycle can function as a template in the next cycle.

[00189] An embodiment of isothermal self-sustained sequence reaction, also sometimes referred to as transcription-mediated amplification or TMA, involves synthesizing single-stranded RNA, single-stranded DNA and double-stranded DNA. The single-stranded RNA is a first template for a first primer, the single-stranded DNA is a second template for a second primer, and the double stranded DNA is a third template for synthesis of a plurality of copies of

the first template. A sequence of the first primer or the second primer is complementary to a sequence of a target nucleic acid and a sequence of the first primer or the second primer is homologous to a sequence of the target nucleic acid. In an embodiment of an isothermal self-sustained sequence reaction, a first cDNA strand is synthesized by extension of the first primer along the target by an enzyme with RNA-dependent DNA polymerase activity, such as a reverse transcriptase. The first primer comprises a polymerase binding sequence (PBS) such as a PBS for a DNA-dependent RNA polymerase, such as T7, T3, or SP6 RNA polymerase. The first primer comprising a PBS is sometimes referred to as a promoter-primer. The first cDNA strand is rendered single-stranded, such as by denaturation or by degradation of the RNA, such as by an RNase H. The second primer then anneals to the first cDNA strand and is extended to form a second cDNA strand by an enzyme with DNA-dependent DNA polymerase activity, such as a reverse transcriptase or a DNA polymerase I. Forming the second cDNA strand renders the cDNA double-stranded, including the PBS. RNA can then be synthesized from the cDNA, which comprises the PBS, by a DNA-dependent RNA polymerase, such as T7, T3, or SP6 RNA polymerase, thereby providing a template for further events (extension of the first primer, rendering the product single-stranded, extension of the second primer, and RNA synthesis).

Geometric amplification occurs because the RNA product can subsequently serve as a template and also because RNA products can be generated repeatedly from a cDNA comprising the PBS.

[00190] An embodiment of RPA can be performed isothermally and employs a recombinase to promote strand invasion of a double-stranded template by forward and reverse primers. The strand invasion capability of a recombinase can create a localized denatured region, thereby obviating the need for thermal denaturation conditions. A DNA synthesis reaction conducted with a recombinase, under RPA conditions, can be performed under isothermal conditions. In some embodiments, RPA is performed using one or more strand displacing polymerases. The 3' ends of the primers are extended, displacing template strands at least in part. Subsequent strand invasion/annealing events, including to previously produced extension products, occur and are followed by extension, resulting in amplification. In some embodiments, recombinase activity is supported by the presence of one or more recombinase accessory proteins, such as a recombinase loading protein and/or single-stranded binding protein.

[00191] In some embodiments, the disclosure relates generally to compositions, and related methods, systems, kits and apparatuses, comprising a nucleic acid synthesis or nucleic acid

amplification reaction (amplification condition) that can be conducted under thermo-cycling or isothermal conditions, or a combination of both types of conditions. For example, the amplification condition can include alternating between thermocycling and isothermal amplification conditions, in any order.

[00192] In some embodiments thermo-cycling amplification conditions comprise a nucleic acid amplification reaction mixture that is subjected to an elevated temperature for a period of time that is sufficient to denature at least about 30-95% of the double-stranded target nucleic acids, and then subjected to a lower temperature for a period of time that is sufficient to permit hybridization between the single-stranded target nucleic acids and any of the primers (e.g., capture primer, reverse solution-phase primer, or fusion primer). In some embodiments, the increase and decrease temperature cycle is repeated at least once.

[00193] In some embodiments isothermal amplification conditions comprise a nucleic acid amplification reaction mixture that is subjected to a temperature variation which is constrained within a limited range during at least some portion of the amplification, including for example a temperature variation is within about 20°C, or about 10°C, or about 5°C, or about 1-5°C, or about 0.1-1°C, or less than about 0.1°C.

[00194] In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 2, 5, 10, 15, 20, 30, 40, 50, 60 or 120 minutes, or longer. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for at least about 2 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 120 minutes or less. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 2 to about 120 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 2 to about 60 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 60 to about 120 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 2 to about 5 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 5 to about 10 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 10 to about 15 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 10 to about 15 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 10 to

about 15 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 15 to about 20 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 20 to about 30 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 30 to about 40 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 40 to about 50 minutes. In some embodiments, an isothermal nucleic acid amplification reaction can be conducted for about 50 to about 60 minutes.

[00195] In some embodiments, an isothermal nucleic acid amplification reaction can be conducted at about 15-30°C, or about 30-45°C, or about 45-60°C, or about 60-75°C, or about 75-90°C, or about 90-93°C, or about 93-99°C.

[00196] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatuses, that further include an enrichment step. In some embodiments, an amplified population of nucleic acids can include an affinity moiety. For example, in conducting any of the nucleic acid synthesis or amplification methods according to the present teachings, a solution-phase/reverse primer that is attached to an affinity moiety (e.g., biotin) can be used to conduct an amplification reaction to produce an amplified population of nucleic acids that are attached to the affinity moiety. In some embodiments, the enrichment step comprises forming an enrichment complex by binding the affinity moiety (which is attached to the amplified population of nucleic acids) with a purification particle (e.g., paramagnetic bead) that is attached to a receptor moiety (e.g., streptavidin). An example of purification particles include MyOne™ Beads from Dynabeads, which are paramagnetic beads attached to streptavidin. In some embodiments, a magnet can be used to separate/remove the enrichment complex from amplified population of nucleic acids that lack the affinity moiety. In some embodiments, the enrichment step can be repeated at least once. In some embodiment, the enrichment step is followed by one or more washing step.

[00197] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatuses that further include at least one washing step. The washing step can be conducted at any time during the workflow for nucleic acid synthesis or amplification. In some embodiments, a washing step can remove excess or unreacted components of the nucleic acid synthesis (e.g., amplification) or enrichment reactions.

[00198] In some embodiments, any of the nucleic acid synthesis or amplification methods, or enrichment steps, according to the present teachings, can be conducted manually or by automation. In some embodiments, any one or any combination of the steps can be conducted manually or by automation, including: (1) forming a single reaction mixture, (2) conducting a nucleic acid amplification reaction, (3) enriching and/or (4) washing. For example, any reagents for a nucleic acid synthesis (e.g., amplification), enrichment or washing, can be deposited into, or removed from, a reaction vessel via manual or automated modes. In some embodiments, reagents for nucleic acid synthesis include any one or any combination of target polynucleotides, particles attached with capture primers, solution-phase primers, fusion primers, other additional primers, enzymes (e.g., polymerases), accessory proteins (e.g., recombinase, recombinase loading protein, single-stranded binding protein, helicase or topoisomerase), nucleotides, divalent cations, binding partners, co-factors and/or buffer.

[00199] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatuses, which further include a sequencing reaction. In some embodiments, any target polynucleotide that has been amplified according to the present teachings can be sequenced.

[00200] In some embodiments, any type of sequencing platform can be employed, including: sequencing by oligonucleotide probe ligation and detection (e.g., SOLiD™ from Life Technologies, WO 2006/084131), probe-anchor ligation sequencing (e.g., Complete Genomics™ or Polonator™), sequencing-by-synthesis (e.g., Genetic Analyzer and HiSeq™, from Illumina), pyrophosphate sequencing (e.g., Genome Sequencer FLX from 454 Life Sciences), ion-sensitive sequencing (e.g., Personal Genome Machine (PGM™) and Ion Proton™ Sequencer, both from Ion Torrent Systems, Inc.), and single molecule sequencing platforms (e.g., HeliScope™ from Helicos™).

[00201] In some embodiments, nucleic acids that have been synthesized, or have been amplified, according to the present teachings can be sequenced by any sequencing method, including sequencing-by-synthesis, ion-based sequencing involving the detection of sequencing byproducts using field effect transistors (e.g., FETs and ISFETs), chemical degradation sequencing, ligation-based sequencing, hybridization sequencing, pyrophosphate detection sequencing, capillary electrophoresis, gel electrophoresis, next-generation, massively parallel sequencing platforms, sequencing platforms that detect hydrogen ions or other sequencing by-

products, and single molecule sequencing platforms. In some embodiments, a sequencing reaction can be conducted using at least one sequencing primer that can hybridize to any portion of the polynucleotide constructs, including a nucleic acid adaptor or a target polynucleotide.

[00202] In some embodiments, the disclosure relates generally to methods, as well as related systems, compositions, kits and apparatuses, for conducting a sequencing reaction on a support having one or more reaction sites coupled to a sensor.

[00203] In some embodiments, any target polynucleotide that is amplified according to the present teachings can be sequenced using methods that detect one or more byproducts of nucleotide incorporation. The detection of polymerase extension by detecting physicochemical byproducts of the extension reaction, can include pyrophosphate, hydrogen ion, charge transfer, heat, and the like, as disclosed, for example, in U.S. Pat. No. 7,948,015 to Rothberg et al.; and Rothberg et al, U.S. Patent Publication No. 2009/0026082, hereby incorporated by reference in their entireties. Other examples of methods of detecting polymerase-based extension can be found, for example, in Pourmand et al, Proc. Natl. Acad. Sci., 103: 6466-6470 (2006); Purushothaman et al., IEEE ISCAS, IV-169-172; Anderson et al, Sensors and Actuators B Chem., 129: 79-86 (2008); Sakata et al., Angew. Chem. 118:2283-2286 (2006); Esfandyapour et al., U.S. Patent Publication No. 2008/01666727; and Sakurai et al., Anal. Chem. 64: 1996-1997 (1992).

[00204] Reactions involving the generation and detection of ions are widely performed. The use of direct ion detection methods to monitor the progress of such reactions can simplify many current biological assays. For example, template-dependent nucleic acid synthesis by a polymerase can be monitored by detecting hydrogen ions that are generated as natural byproducts of nucleotide incorporations catalyzed by the polymerase. Ion-sensitive sequencing (also referred to as “pH-based” or “ion-based” nucleic acid sequencing) exploits the direct detection of ionic byproducts, such as hydrogen ions, that are produced as a byproduct of nucleotide incorporation. In one exemplary system for ion-based sequencing, the nucleic acid to be sequenced can be captured in a microwell, and nucleotides can be flowed across the well, one at a time, under nucleotide incorporation conditions. The polymerase incorporates the appropriate nucleotide into the growing strand, and the hydrogen ion that is released can change the pH in the solution, which can be detected by an ion sensor that is coupled with the well. This technique does not require labeling of the nucleotides or expensive optical components, and

allows for far more rapid completion of sequencing runs. Examples of such ion-based nucleic acid sequencing methods and platforms include the Ion Torrent PGM™ or Proton™ sequencer (Ion Torrent™ Systems, Thermo Fisher Scientific).

[00205] In some embodiments, target polynucleotides produced using the methods, systems and kits of the present teachings can be used as a substrate for a biological or chemical reaction that is detected and/or monitored by a sensor including a field-effect transistor (FET). In various embodiments the FET is a chemFET or an ISFET. A “chemFET” or chemical field-effect transistor, is a type of field effect transistor that acts as a chemical sensor. It is the structural analog of a MOSFET transistor, where the charge on the gate electrode is applied by a chemical process. An “ISFET” or ion-sensitive field-effect transistor, is used for measuring ion concentrations in solution; when the ion concentration (such as H⁺) changes, the current through the transistor will change accordingly. A detailed theory of operation of an ISFET is given in "Thirty years of ISFETOLOGY: what happened in the past 30 years and what may happen in the next 30 years," P. Bergveld, Sens. Actuators, 88 (2003), pp. 1-20.

[00206] In some embodiments, the FET may be a FET array. As used herein, an “array” is a planar arrangement of elements such as sensors or wells. The array may be one or two dimensional. A one dimensional array can be an array having one column (or row) of elements in the first dimension and a plurality of columns (or rows) in the second dimension. The number of columns (or rows) in the first and second dimensions may or may not be the same. The FET or array can comprise 10², 10³, 10⁴, 10⁵, 10⁶, 10⁷ or more FETs.

[00207] In some embodiments, one or more microfluidic structures can be fabricated above the FET sensor array to provide for containment and/or confinement of a biological or chemical reaction. For example, in one implementation, the microfluidic structure(s) can be configured as one or more wells (or microwells, or reaction chambers, or reaction wells, as the terms are used interchangeably herein) disposed above one or more sensors of the array, such that the one or more sensors over which a given well is disposed detect and measure analyte presence, level, and/or concentration in the given well. In some embodiments, there can be a 1:1 correspondence of FET sensors and reaction wells. Exemplary embodiments of FET sensor arrays can be found in U.S. patent Nos. 7,948,015; 8,262,900; 8,776,573; 8,208,712.

[00208] Microwells or reaction chambers are typically hollows or wells having well-defined shapes and volumes which can be manufactured into a substrate and can be fabricated using

conventional microfabrication techniques, e.g. as disclosed in the following references: Doering and Nishi, Editors, Handbook of Semiconductor Manufacturing Technology, Second Edition (CRC Press, 2007); Saliterman, Fundamentals of BioMEMS and Medical Microdevices (SPIE Publications, 2006); Elwenspoek et al, Silicon Micromachining (Cambridge University Press, 2004); and the like. Examples of configurations (e.g. spacing, shape and volumes) of microwells or reaction chambers are disclosed in Rothberg et al, U.S. patent publication 2009/0127589; Rothberg et al, U.K. patent application GB24611127.

[00209] In some embodiments, the biological or chemical reaction can be performed in a solution or a reaction chamber that is in contact with, operatively coupled, or capacitively coupled to a FET such as a chemFET or an ISFET. The FET (or chemFET or ISFET) and/or reaction chamber can be an array of FETs or reaction chambers, respectively.

[00210] In some embodiments, a biological or chemical reaction can be carried out in a two-dimensional array of reaction chambers, wherein each reaction chamber can be coupled to a FET, and each reaction chamber is no greater than $10\ \mu\text{m}^3$ (i.e., 1 pL) in volume. In some embodiments each reaction chamber is no greater than 0.34 pL, 0.096 pL or even 0.012 pL in volume. A reaction chamber can optionally be no greater than 2, 5, 10, 15, 22, 32, 42, 52, 62, 72, 82, 92, or 102 square microns in cross-sectional area at the top. Preferably, the array has at least 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , or more reaction chambers. In some embodiments, at least one of the reaction chambers is operatively coupled to at least one of the FETs.

[00211] FET arrays as used in various embodiments according to the disclosure can be fabricated according to conventional CMOS fabrications techniques, as well as modified CMOS fabrication techniques and other semiconductor fabrication techniques beyond those conventionally employed in CMOS fabrication. Additionally, various lithography techniques can be employed as part of an array fabrication process.

[00212] Exemplary FET arrays suitable for use in the disclosed methods, as well as microwells and attendant fluidics, and methods for manufacturing them, are disclosed, for example, in U.S. Patent Publication No. 20100301398; U.S. Patent Publication No. 20100300895; U.S. Patent Publication No. 20100300559; U.S. Patent Publication No. 20100197507, U.S. Patent Publication No. 20100137143; U.S. Patent Publication No. 20090127589; and U.S. Patent Publication No. 20090026082, which are incorporated by reference in their entireties.

[00213] In one aspect, the disclosed methods, compositions, systems, apparatuses and kits can be used for carrying out label-free nucleic acid sequencing, and in particular, ion-based nucleic acid sequencing. The concept of label-free detection of nucleotide incorporation has been described in the literature, including the following references that are incorporated by reference: Rothberg et al, U.S. patent publication 2009/0026082; Anderson et al, Sensors and Actuators B Chem., 129: 79-86 (2008); and Pourmand et al, Proc. Natl. Acad. Sci., 103: 6466-6470 (2006). Briefly, in nucleic acid sequencing applications, nucleotide incorporations are determined by measuring natural byproducts of polymerase-catalyzed extension reactions, including hydrogen ions, polyphosphates, PPi, and Pi (e.g., in the presence of pyrophosphatase). Examples of such ion-based nucleic acid sequencing methods and platforms include the Ion Torrent PGM™ or Proton™ sequencer (Ion Torrent™ Systems, Thermo Fisher Scientific).

[00214] In some embodiments, the disclosure relates generally to methods for sequencing nucleic acids that have been amplified by the teachings provided herein. In one exemplary embodiment, the disclosure relates generally to a method for obtaining sequence information from polynucleotides, comprising: (a) amplifying target polynucleotides; and (b) sequencing the amplified target polynucleotides by performing template-dependent nucleic acid synthesis using at least one of the amplified target polynucleotides produced during step (a) as a template. The amplifying can optionally be performed according to any of the amplification methods described herein.

[00215] In some embodiments, the template-dependent synthesis includes incorporating one or more nucleotides in a template-dependent fashion into a newly synthesized nucleic acid strand.

[00216] Optionally, the methods can further include producing one or more ionic byproducts of such nucleotide incorporation.

[00217] In some embodiments, the methods can further include detecting the incorporation of the one or more nucleotides into the sequencing primer. Optionally, the detecting can include detecting the release of hydrogen ions.

[00218] In another embodiment, the disclosure relates generally to a method for sequencing a nucleic acid, comprising: (a) amplifying target polynucleotides to generate at least one particle attached with a substantially monoclonal polynucleotide population containing a portion of one of the target polynucleotides, according to the teachings disclosed herein; and (b) disposing the

particles into a reaction chambers, wherein one or more of the reaction chambers are in contact with a field effect transistor (FET). Optionally, the method further includes contacting the amplified nucleic acids which are disposed into one of the reaction chambers, with a polymerase thereby synthesizing a new nucleic acid strand by sequentially incorporating one or more nucleotides into a nucleic acid molecule. Optionally, the method further includes generating one or more hydrogen ions as a byproduct of such nucleotide incorporation. Optionally, the method further includes detecting the incorporation of the one or more nucleotides by detecting the generation of the one or more hydrogen ions using the FET.

[00219] In some embodiments, the detecting includes detecting a change in voltage and/or current at the at least one FET within the array in response to the generation of the one or more hydrogen ions.

[00220] In some embodiments, the FET can be selected from the group consisting of: ion-sensitive FET (isFET) and chemically-sensitive FET (chemFET).

[00221] In some embodiments, the disclosure relates generally to methods (and related compositions, systems, kits and apparatuses) for nucleic acid sequencing, comprising identifying a series of contiguous nucleotides in a nucleic acid template according to any of the methods disclosed herein.

[00222] One exemplary system involving sequencing via detection of ionic byproducts of nucleotide incorporation is the Ion Torrent PGM™ or Proton™ sequencer (Thermo Fisher Scientific), which is an ion-based sequencing system that sequences nucleic acid templates by detecting hydrogen ions produced as a byproduct of nucleotide incorporation. Typically, hydrogen ions are released as byproducts of nucleotide incorporations occurring during template-dependent nucleic acid synthesis by a polymerase. The Ion Torrent PGM™ or Proton™ sequencer detects the nucleotide incorporations by detecting the hydrogen ion byproducts of the nucleotide incorporations. The Ion Torrent PGM™ or Proton™ sequencer can include a plurality of nucleic acid templates to be sequenced, each template disposed within a respective sequencing reaction well in an array. The wells of the array can each be coupled to at least one ion sensor that can detect the release of H⁺ ions or changes in solution pH produced as a byproduct of nucleotide incorporation. The ion sensor comprises a field effect transistor (FET) coupled to an ion-sensitive detection layer that can sense the presence of H⁺ ions or changes in solution pH. The ion sensor can provide output signals indicative of nucleotide incorporation

which can be represented as voltage changes whose magnitude correlates with the H^+ ion concentration in a respective well or reaction chamber. Different nucleotide types can be flowed serially into the reaction chamber, and can be incorporated by the polymerase into an extending primer (or polymerization site) in an order determined by the sequence of the template. Each nucleotide incorporation can be accompanied by the release of H^+ ions in the reaction well, along with a concomitant change in the localized pH. The release of H^+ ions can be registered by the FET of the sensor, which produces signals indicating the occurrence of the nucleotide incorporation. Nucleotides that are not incorporated during a particular nucleotide flow may not produce signals. The amplitude of the signals from the FET can also be correlated with the number of nucleotides of a particular type incorporated into the extending nucleic acid molecule thereby permitting homopolymer regions to be resolved. Thus, during a run of the sequencer multiple nucleotide flows into the reaction chamber along with incorporation monitoring across a multiplicity of wells or reaction chambers can permit the instrument to resolve the sequence of many nucleic acid templates simultaneously. Further details regarding the compositions, design and operation of the Ion Torrent PGM™ or Proton™ sequencer can be found, for example, in U.S. Patent Application Ser. No. 12/002781, now published as U.S. Patent Publication No. 2009/0026082; U.S. Patent Application Ser. No. 12/474897, now published as U.S. Patent Publication No. 2010/0137143; and U.S. Patent Application Ser. No. 12/492844, now published as U.S. Patent Publication No. 2010/0282617, all of which applications are incorporated by reference herein in their entireties.

[00223] In a typical embodiment of ion-based nucleic acid sequencing, nucleotide incorporations can be detected by detecting the presence and/or concentration of hydrogen ions generated by polymerase-catalyzed extension reactions. In one embodiment, templates, optionally pre-bound to a sequencing primer and/or a polymerase, can be loaded into reaction chambers (such as the microwells disclosed in Rothberg et al, cited herein), after which repeated cycles of nucleotide addition and washing can be carried out. In some embodiments, such templates can be attached as clonal populations to a solid support, such as particles, bead, or the like, and said clonal populations are loaded into reaction chambers.

[00224] In another embodiment, the templates, optionally bound to a polymerase, are distributed, deposited or positioned to different sites of the array. The sites of the array include

primers and the methods can include hybridizing different templates to the primers within different sites.

[00225] In each addition step of the cycle, the polymerase can extend the primer by incorporating added nucleotide only if the next base in the template is the complement of the added nucleotide. If there is one complementary base, there is one incorporation, if two, there are two incorporations, if three, there are three incorporations, and so on. With each such incorporation there is a hydrogen ion released, and collectively a population of templates releasing hydrogen ions changes the local pH of the reaction chamber. The production of hydrogen ions is monotonically related to the number of contiguous complementary bases in the template (as well as the total number of template molecules with primer and polymerase that participate in an extension reaction). Thus, when there are a number of contiguous identical complementary bases in the template (i.e. a homopolymer region), the number of hydrogen ions generated, and therefore the magnitude of the local pH change, can be proportional to the number of contiguous identical complementary bases. If the next base in the template is not complementary to the added nucleotide, then no incorporation occurs and no hydrogen ion is released. In some embodiments, after each step of adding a nucleotide, an additional step can be performed, in which an unbuffered wash solution at a predetermined pH is used to remove the nucleotide of the previous step in order to prevent misincorporations in later cycles. In some embodiments, the after each step of adding a nucleotide, an additional step can be performed wherein the reaction chambers are treated with a nucleotide-destroying agent, such as apyrase, to eliminate any residual nucleotides remaining in the chamber, which may result in spurious extensions in subsequent cycles.

[00226] In one exemplary embodiment, different kinds of nucleotides are added sequentially to the reaction chambers, so that each reaction can be exposed to the different nucleotides one at a time. For example, nucleotides can be added in the following sequence: dATP, dCTP, dGTP, dTTP, dATP, dCTP, dGTP, dTTP, and so on; with each exposure followed by a wash step. The cycles may be repeated for 50 times, 100 times, 200 times, 300 times, 400 times, 500 times, 750 times, or more, depending on the length of sequence information desired.

[00227] In some embodiments, sequencing can be performed according to the user protocols supplied with the PGMTM or ProtonTM sequencer. Example 3 provides one exemplary protocol

for ion-based sequencing using the Ion Torrent PGM™ sequencer (Ion Torrent™ Systems, Thermo Fisher Scientific).

[00228] In some embodiments, the disclosure relates generally to methods for sequencing a population of template polynucleotides, comprising: (a) generating a plurality of amplicons by clonally amplifying a plurality of target polynucleotides onto a plurality of particles, wherein the amplifying is performed within a single continuous phase of a reaction mixture and wherein at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of the resulting amplicons are substantially monoclonal in nature. In some embodiments, a sufficient number of substantially monoclonal amplicons are produced in a single amplification reaction to generate at least 100 MB, 200MB, 300 MB, 400 MB, 500MB, 750 MB, 1GB or 2 GB of AQ20 sequencing reads on an Ion Torrent PGM™ 314, 316 or 318 sequencer. The term “AQ20 and its variants, as used herein, refers to a particular method of measuring sequencing accuracy in the Ion Torrent PGM™ sequencer. Accuracy can be measured in terms of the Phred-like Q score, which measures accuracy on logarithmic scale that: Q10 = 90%, Q20= 99%, Q30 = 99.9%, Q40 = 99.99%, and Q50 = 99.999%. For example, in a particular sequencing reaction, accuracy metrics can be calculated either through prediction algorithms or through actual alignment to a known reference genome. Predicted quality scores (“Q scores”) can be derived from algorithms that look at the inherent properties of the input signal and make fairly accurate estimates regarding if a given single base included in the sequencing “read” will align. In some embodiments, such predicted quality scores can be useful to filter and remove lower quality reads prior to downstream alignment. In some embodiments, the accuracy can be reported in terms of a Phred-like Q score that measures accuracy on logarithmic scale such that: Q10 = 90%, Q17 = 98%, Q20= 99%, Q30 = 99.9%, Q40 = 99.99%, and Q50 = 99.999%. In some embodiments, the data obtained from a given polymerase reaction can be filtered to measure only polymerase reads measuring “N” nucleotides or longer and having a Q score that passes a certain threshold, e.g., Q10, Q17, Q100 (referred to herein as the “NQ17” score). For example, the 100Q20 score can indicate the number of reads obtained from a given reaction that are at least 100 nucleotides in length and have Q scores of Q20 (99%) or greater. Similarly, the 200Q20 score can indicate the number of reads that are at least 200 nucleotides in length and have Q scores of Q20 (99%) or greater.

[00229] In some embodiments, the accuracy can also be calculated based on proper alignment using a reference genomic sequence, referred to herein as the “raw” accuracy. This is single pass accuracy, involving measurement of the “true” per base error associated with a single read, as opposed to consensus accuracy, which measures the error rate from the consensus sequence which is the result of multiple reads. Raw accuracy measurements can be reported in terms of “AQ” scores (for aligned quality). In some embodiments, the data obtained from a given polymerase reaction can be filtered to measure only polymerase reads measuring “N” nucleotides or longer having a AQ score that passes a certain threshold, e.g., AQ10, AQ17, AQ100 (referred to herein as the “NAQ17” score). For example, the 100AQ20 score can indicate the number of reads obtained from a given polymerase reaction that are at least 100 nucleotides in length and have AQ scores of AQ20 (99%) or greater. Similarly, the 200AQ20 score can indicate the number of reads that are at least 200 nucleotides in length and have AQ scores of AQ20 (99%) or greater.

[00230] In some embodiments, the disclosure relates generally to methods, and related compositions, systems, kits and apparatuses, that further comprise depositing one or more particles onto a surface such as a detection area of a nucleic acid sequencing instrument or a location where nucleic acid sequencing reactions occur, wherein at least one particle is prepared according to the present teachings and is attached with a substantially monoclonal polynucleotide population containing a portion of one of the target polynucleotides.

[00231] In some embodiments, the detection area of a nucleic acid sequencing instrument or the location where nucleic acid sequencing reactions occur includes a planar surface, flowcell, channel, reaction chamber, or an array of reaction chambers.

[00232] In some embodiments, reagents can be delivered to the detection area of a nucleic acid sequencing instrument, or delivered to the location where nucleic acid sequencing reactions occur, to conduct a nucleic acid sequencing reaction.

[00233] In some embodiments, nucleic acid sequencing reactions can be conducted by flowing sequencing reagents onto a planar surface, flowcell, channel, reaction chamber, or an array of reaction chambers having the deposited particles which are attached with a substantially monoclonal polynucleotide population containing a portion of one of the target polynucleotides.

[00234] In some embodiments, sequencing reagents can include any combination of polymerases, nucleotides, cations, and/or buffers. In some embodiments, sequencing reactions

include: chemical degradation sequencing reactions; enzyme cascade reactions; chain-termination sequencing reactions; sequencing-by-synthesis reactions; sequencing-by-ligation reactions; and ion-detection-based reactions.

[00235] In some embodiments, the particles can be deposited into reaction chambers organized in an array, where the reaction chambers are coupled with at least one ion-sensitive sensor. Typically, a reaction chamber can hold one particle (e.g., which is attached with a population of polynucleotides). In some embodiments, a reaction chamber can also hold particles conjugated with enzymes (e.g., enzyme-beads). The enzyme-beads can be microparticles coated with one or more types of enzymes for conducting an enzyme cascade reaction. The enzyme cascade can be a pyrophosphate-sulfurylase-luciferase enzyme cascade.

[00236] In some embodiments, a sequencing reaction comprises: sequencing by oligonucleotide probe ligation and detection (e.g., SOLiDTM), probe-anchor ligation sequencing (e.g., Complete Genomics or PolonatorTM), sequence-by-synthesis (e.g., Illumina), pyrophosphate sequencing (e.g., 454 Life Sciences), ion-sensitive sequencing (e.g., Personal Genome Machine (PGMTM) and Ion ProtonTM Sequencer, both from Ion Torrent Systems, Inc.) and single molecule sequencing platforms (e.g., HelicosTM).

[00237] In some embodiments, the disclosure relates generally to compositions, as well as related systems, methods, kits and apparatuses, comprising a single reaction mixture that contains an emulsion to provide compartmentalization for separately amplifying different target polynucleotides. In some embodiments, the emulsion comprises two immiscible liquid phases. In some embodiments, two immiscible liquid phases are mixed together to make the emulsion. In some embodiments, one of the liquid phases is dispersed in the other. Optionally, the emulsion comprises a discontinuous hydrophilic phase and a continuous hydrophobic phase. Optionally, the discontinuous hydrophilic phase is surrounded by the continuous hydrophobic phase. Optionally, the emulsion comprises at least one hydrophilic phase compartment (e.g., droplet or micro-reactor) surrounded by a continuous hydrophobic phase. Optionally, the discontinuous hydrophilic phase provides a compartment. Optionally, the emulsion comprises a plurality of hydrophilic phase droplets and a continuous hydrophobic phase. Optionally, the emulsion comprises a plurality of aqueous droplets and a continuous hydrophobic phase. Optionally, the emulsion comprises at least one aqueous droplet. Optionally, the at least one aqueous droplet includes one or more particles. Optionally, the at least one aqueous droplet

includes one or more different target polynucleotides. Optionally, the emulsion includes at least one aqueous droplet that includes one or more particles of the first type, the second type, or particles of both the first and second type. Optionally, the emulsion includes at least one aqueous droplet that includes one or more different target polynucleotides.

[00238] Optionally, the emulsion comprises a mixture of an aqueous liquid and a water-immiscible organic liquid. Optionally, the emulsion comprises at least one anionic, cationic or non-ionic surfactant. Optionally, the emulsion can have a droplet-type dispersion comprising oil-in-water, water-in-oil, or a bicontinuous microemulsion.

[00239] In some embodiments, the water immiscible organic liquid comprises an oil. In some embodiments, the oil can be from a natural source, including animal (e.g., tallow or lard), fish (e.g., fish oil), shark, seeds, nuts or plants (e.g., vegetable oils). Optionally, the oil can be from derived from petroleum, including mineral oils. Optionally, the oil comprises a fluorochemical oil, polyalphaolefin or ester oil.

[00240] In some embodiments, the surfactant includes small molecule surfactants, polymeric surfactants, triblock co-polymer surfactants or non-ionic block copolymer surfactants. Optionally, the surfactant comprises a sorbitan oleate or a silicone surfactant.

[00241] Optionally, the hydrophilic phase compartment can contain at least two different types of particles. Optionally, the hydrophilic phase compartment can contain at least two different types of target polynucleotides.

[00242] EXAMPLES

[00243] The following examples are provided to illustrate certain disclosed embodiments and are not to be construed as limiting the scope of this disclosure in any way.

[00244] Example 1:

[00245] Amplification and sequencing of a target polynucleotide sequence from a blood sample without need for attaching a universal adaptor sequence.

[00246] A blood sample that is optionally processed, e.g., to remove undesired material such as red blood cells and insoluble matter, is contacted with a set of particles having attached capture primers specific for a target polynucleotide sequence present in the sample and with solution phase primers specific for the target polynucleotide sequence and oppositely oriented to the capture primers, thus forming a single reaction mixture. No attachment of a universal adaptor sequence is necessary. Reagents for amplification are added and the mixture is subjected

to amplification conditions. Amplification produces a substantially monoclonal polynucleotide population attached to one or more of the set of particles.

[00247] A sequencing reaction is optionally performed using the substantially monoclonal polynucleotide population as a template to obtain sequence data for the target polynucleotide sequence of the subject.

[00248] Example 2:

[00249] Amplification and sequencing of a target polynucleotide lacking a universal adaptor sequence.

[00250] A plurality of target polynucleotides lacking a universal adaptor sequence is contacted in a single continuous liquid phase with a set of particles having attached capture primers specific for the target polynucleotide and with solution phase primers specific for the target polynucleotide and oppositely oriented to the capture primers, thus forming a single reaction mixture. Reagents for amplification are added and the mixture is subjected to amplification conditions. Amplification produces a substantially monoclonal polynucleotide population attached to one or more of the set of particles.

[00251] A sequencing reaction is optionally performed using the substantially monoclonal polynucleotide population as a template to obtain sequence data for the target polynucleotide sequence.

[00252] Example 3:

[00253] Amplification and sequencing of multiple target polynucleotide sequences from a blood sample without need for attaching a universal adaptor sequence.

[00254] A blood sample that is optionally processed, e.g., to remove undesired material such as red blood cells and insoluble matter, is contacted with at least first and second sets of particles. The particles of the first set have attached first capture primers specific for a first target polynucleotide sequence present in the sample, the particles of the second set have attached second capture primers specific for a second target polynucleotide sequence present in the sample, and so on. The contacting is carried out so as to form hybridized complexes of capture primers and target polynucleotides. At least first and second solution-phase primers specific for the first and second target polynucleotide sequences, respectively, are also contacted with the sample. Thus, a single reaction mixture is formed. No attachment of a universal adaptor sequence to polynucleotides of the sample is necessary. The mixture is subjected to amplification

conditions including addition of amplification reagents such as nucleotide triphosphates, a polymerase, and appropriate buffer, salt, etc. Amplification produces at least a first substantially monoclonal polynucleotide population attached to one or more of the first set of particles and a second substantially monoclonal polynucleotide population attached to one or more of the second set of particles.

[00255] A sequencing reaction is optionally performed using substantially monoclonal polynucleotide populations as templates to obtain sequence data for at least the first and second target polynucleotide sequences of the subject. The sequencing reactions can be performed in parallel, e.g., using a sequencer with a plurality of reaction sites coupled to sensors configured to generate sequence data from a substantially monoclonal polynucleotide population attached to a particle.

[00256] Example 4:

[00257] PCR.

[00258] Any of examples 1, 2, and 3 is performed in which the primers are suitable for PCR amplification of the target sequence(s) and the amplification conditions include a thermostable DNA-dependent DNA polymerase and thermocycling.

[00259] Example 5:

[00260] Isothermal self-sustained sequence reaction.

[00261] Any of examples 1, 2, and 3 is performed in which the primers are suitable for amplification via an isothermal self-sustained sequence reaction (e.g., including a promoter-primer) of the target sequence(s) and the amplification conditions include the presence of RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and DNA-dependent RNA polymerase activities and optionally RNase H activity. The amplification can be carried out under substantially isothermal conditions.

[00262] Example 6:

[00263] Recombinase-mediated amplification.

[00264] Any of examples 1, 2, and 3 is performed in which the primers are suitable for amplification via a recombinase-mediated amplification reaction of the target sequence(s) and the amplification conditions include the presence of a recombinase (which can catalyze strand invasion of the target polynucleotide(s) by the appropriate primers) and a DNA-dependent DNA polymerase. The amplification conditions optionally further include the presence of a

recombinase loading protein and/or single-stranded binding protein. The amplification can be carried out under substantially isothermal conditions.

[00265] Example 7:

[00266] Sequencing with a polynucleotide-specific primer.

[00267] Any of examples 4, 5, and 6 is performed in which a polynucleotide-specific primer or primers is (are) used to prime a sequencing reaction following generation of the substantially monoclonal population(s).

[00268] Example 8:

[00269] Sequencing with a universal primer.

[00270] Any of examples 4, 5, and 6 is performed in which one or more capture or solution-phase primers comprise a universal sequence in addition to a sequence specific for a target polynucleotide. A universal primer that hybridizes to the complement of the universal sequence is used to prime a sequencing reaction following generation of the substantially monoclonal population(s).

[00271] Example 9:

[00272] Procedure for preparing beads having polynucleotide-specific capture primers attached thereon.

[00273] Beads attached with a plurality of universal sequences and polynucleotide-specific capture primer sequences were prepared, starting with beads attached with a plurality of B capture primers (universal sequence), and enzymatically ligating the B capture primers to polynucleotide-specific capture primers using a single-stranded fusion primer as a splint to increase the yield of ligation products. The various polynucleotide-specific capture primers contained a phosphate moiety at their 5' ends. The 3' portion of the fusion primers contained a sequence complementary to the B sequence, and the 5' portion contained a sequence complementary to the desired capture sequence.

[00274] For example, (i) a first set of beads was prepared by ligating the B capture primers on the B beads to a plurality of 268 capture primers using fusion primers having sequences complementary to the B and 268 sequences; (ii) a second set of beads was prepared by ligating the B capture primers on the B beads to a plurality of 241 capture primers using fusion primers having sequences complementary to the B and 241 sequences; and (iii) a third set of beads was

prepared by ligating the B capture primers on the B beads to a plurality of 058 capture primers using fusion primers having sequences complementary to the B and 058 sequences.

[00275] The ligation reaction was set up in 0.2 mL PCR tubes as follows. A fast hybridization reaction was set up containing: (a) 15.9 uL of B beads (concentration 126 million beads/uL) for a final amount of about 2 billion B beads; (b) 2 uL of fusion primers (concentration 1 mM) for a final amount of about 20 uM; (c) 76 uL ligation buffer. The fast hybridization reaction was conducted at 98 °C for 2 minutes, and 37 °C for 2 minutes. After the fast hybridization reaction, the following were added: 2 uL of the capture primers (268, 241 or 058) (1 mM) for a final amount of about 20 uM; and 4 uL T4 ligase. The ligation reaction was incubated at room temperature for about 30 minutes. The ligation reaction was transferred to a 1.5 mL tube. The yield of the ligation reaction throughout the workflow was monitored by taking aliquots for guava analysis. 900 uL of nuclease-free water was added to the tube, the tube was vortexed well, and spun down at about 21,000 rcf (rotation speed of the centrifuge) for 8 minutes to pellet the beads. The supernatant was removed down to about 100 uL volume. The remaining sample in the tube was vortexed well. An aliquot was removed for guava analysis. 500 uL of 1 mM NaOH was added to the sample remaining in the tube. The sample was vortexed well, and incubated at 95 °C for 5 minutes, then snap cooled on ice for 2 minutes. The sample was spun down at about 21,000 rcf for 8 minutes. Again, the supernatant was removed down to about 100 uL volume. The remaining sample in the tube was vortexed well. An aliquot was removed for a second guava analysis. 900 uL of nuclease-free water was added to the tube, the tube was vortexed well, and spun down at about 21,000 rcf for 8 minutes. The supernatant was removed down to about 100 uL volume. The remaining sample in the tube was vortexed well. An aliquot was removed for a third guava analysis. 900 uL of nuclease-free water was added to the tube, the tube was vortexed well, and spun down at about 21,000 rcf for 8 minutes. The supernatant was removed down to about 100 uL volume. The remaining sample in the tube was vortexed well. An aliquot was removed for a fourth guava analysis. 900 uL of nuclease-free water was added to the tube, the tube was vortexed well, and spun down at about 21,000 rcf for 8 minutes. The supernatant was removed down to about 100 uL volume. The remaining sample in the tube was vortexed well. An aliquot was removed for a fifth (and final) guava analysis.

[00276] Example 10:

[00277] Procedure for amplifying template molecules with primer-attached beads under isothermal conditions with a recombinase.

[00278] The recombinase polymerase amplification (RPA) reaction was conducted using the bead ligation products, which were prepared as describe in Example 9 above. The recombinase source was from a dehydrated pellet from an Ion PGM Template IA Reagents 500 kit, catalog No. A24619, which is part of an Ion ReproSeq™ PGS Kits, from Thermo Fisher Scientific. Dehydrated pellets in the kit contain uvsX recombinase, uvsY recombinase loading protein, gp32 protein, Bsu DNA polymerase, dNTPs, ATP, phosphocreatine and creatine kinase.

[00279] The RPA reaction was set up as follows: (a) 17 uL of the bead ligation product (total beads approximately 100 million); (b) 5 uL of the target polynucleotide (either DH10b genomic DNA or PCR amplicons containing specific insert sequences) (concentration 1 pM) for a total of about 0.03 mM target polynucleotide; (c) 720 uL of RPA rehydration solution (from the Ion PGM Template IA Reagents 500 kit, catalog No. A24619); and (d) one dehydrated RPA pellet. These reagents were mixed well. The tube contained approximately 750 uL, which was aliquoted into 8 separate samples of 93 uL each.

[00280] For each of the 93 uL aliquots, the following was added: 1 uL of reverse PCR primers (concentration 10 uM) for a total amount of about 62 nM, and 40 uL of start solution (10 mM Tris, pH 8.3 and 54 mM magnesium acetate), and water to make a total volume of 161 uL. The tubes were incubated at 40 °C for 30 minutes. 80 uL of 100 mM EDTA was added to stop the reaction. At this point, the beads are attached (templated) with the target polynucleotides. Alternatively, when an Ion-compatible library is used as the polynucleotide template in this procedure, A-primers (in a mixture with or without biotinylated A-primers) can be used as reverse primers.

[00281] **Example 11:**

[00282] Procedure for washing the templated beads.

[00283] The templated beads (prepared as described in Example 10 above) were subjected to washing and enrichment procedures as follows. The beads were spun at about 21,000 rcf for 8 minutes. The supernatant was removed down to about 100 uL volume. The remaining sample in the tube was vortexed well. An aliquot was removed for guava analysis. 900 uL of nuclease-free water was added and mixed well. 100 uL of paramagnetic streptavidin MyOne Beads (from Dynabeads) was added, and the beads were vortexed 30 seconds, and spun for 2 seconds. The

beads were incubated at room temperature for 2 minutes. The tube was placed on a magnet for 2 minutes. The supernatant was carefully removed, then the tubes were removed from the magnet. 1000 uL of 3 mM SDS was added to the beads, and vortexed for 30 seconds, and spun for 2 seconds. The tube was placed on a magnet for 2 minutes. The supernatant was carefully removed, then the tubes were removed from the magnet. 200 uL of fresh melt-off solution (1 M NaOH and bulk Tween from Enzymatics, CMB0087) was added, and vortexed for 30 seconds, and spun for 2 seconds. The tube was again incubated at room temperature for 2 minutes. The tube was again placed on a magnet for 2 minutes. The supernatant was transferred to a fresh 0.2 mL tube, and spun at about 21,000 rcf for 8 minutes. The supernatant was removed (as much as possible down to approximately 10-15 uL). 90 uL of nuclease-free water was added, and the tube was vortexed well. A sample was removed for guava analysis. The templated beads were loaded onto an Ion Torrent Proton I sequencing chip, and the template polynucleotides were sequenced.

[00284] Example 12:

[00285] Procedure for amplifying template molecules with two sets of primer-attached beads under isothermal conditions with a recombinase.

[00286] Two sets of beads were prepared. Each set of beads included a plurality of oligonucleotides attached to the bead by their 5' ends, and the oligonucleotides contained a universal-B sequence and either a first or second polynucleotide-specific capture primer sequence.

[00287] A recombinase polymerase amplification (RPA) reaction was conducted using the recombinase from a dehydrated pellet, and the Rehydration Buffer, from an Ion PGM Template IA Reagents 500 kit, catalog No. A24619, which is part of an Ion ReproSeq™ PGS Kits, from Thermo Fisher Scientific. Dehydrated pellets in the kit contain uvsX recombinase, uvsY recombinase loading protein, gp32 protein, Bsu DNA polymerase, dNTPs, ATP, phosphocreatine and creatine kinase.

[00288] Seven replicate RPA reactions were prepared for each bead set. The RPA reaction was set up as follows: the Ion PGM Template IA Rehydration Buffer was inverted to mix, and 720 uL was used to rehydrate the Ion PGM Template IA Pellet. The following reagents were added to the rehydrated pellet: (a) approximately 50 million each of the first and second beads; (b) 6 uL of the target polynucleotide (concentration 10 pM); (c) 0.55 uL of 500 mM potassium

acetate; (d) a final concentration of 5 nM of each of a first and second polynucleotide-specific solution-phase primers (each carrying a universal A-sequence at the 5' end and either a first or second polynucleotide-specific region at the 3' end); (e) 1.6 uL of biotinylated soluble primers carrying a universal-A sequence; and (f) enough water (nuclease-free) to make a 20 uL volume. The mixture was vortexed and centrifuged, then iced. 94 uL of the rehydrated pellet was added, and the contents was mixed by vortexing and centrifuged. 45 uL of the Ion PGM Template IA Start Solution (from the Ion ReproSeq™ PGS Kit) was added, and the contents were mixed by pulse-vortexing ten times, then centrifuged, and iced. The reaction was incubated at 40 °C for 40 minutes. The reaction was stopped by addition of 80 uL of 100 mM EDTA.

[00289] The template beads were enriched as follows. Enough water (nuclease-free) was added to make 1 mL. 200 uL of MyOne Streptavidin beads (from Dynabeads) were added. The contents were vortexed and centrifuged, then placed on a rotator for 15 minutes at room temperature. The tubes were briefly centrifuged, then placed on a magnet for 2 minutes at room temperature. The supernatant was carefully removed, then the tubes were removed from the magnet. 1000 uL of 3 mM of SDS was added to the beads, and vortexed to mix, then centrifuged. The tubes were placed on the magnet for 2 minutes at room temperature. The supernatant was carefully removed, then the tubes were removed from the magnet. 400 uL of freshly-prepared Melt-off was added (Melt-off: 1250 uL of 1 M NaOH and 8750 uL of bulk Tween Kit solution), and the tubes were vortexed to mix, then centrifuged. The tubes were incubated at room temperature for 5 minutes, then placed on the magnet for 2 minutes. The supernatant was removed and transferred to a fresh tube. In the fresh tube, enough water (nuclease-free) was added to make 1.2 mL volume. The tube was vortexed, then centrifuged at 15,500 rcf for 8 minutes. The supernatant was removed (down to ~ 25 uL). 25 uL of water (nuclease-free) was added to the tube, and the contents vortexed to mix.

[00290] The 7 replicates of each bead set were pooled, and centrifuged at 15,500 rcf for 5 minutes. The supernatant was removed to down to ~ 9 uL. 15 uL of sequencing primer and 10 uL of annealing buffer were added, and the contents mixed by pipetting up and down, then vortexing, and then briefly centrifuged. The tube was placed in a thermo-cycler: 98 °C for 2 minutes, and 36 °C for 2 minutes. Both sets of templated beads were loaded onto an Ion Torrent P0 sequencing chip, and the template polynucleotides were sequenced.

WHAT IS CLAIMED

1. A method for amplifying nucleic acids, comprising:
 - a) forming a single reaction mixture by contacting, within a single continuous liquid phase,
 - (i) a plurality of target polynucleotides lacking a universal adaptor sequence,
 - (ii) a first set of beads having a plurality of a first polynucleotide-specific capture primer attached thereon,
 - (iii) a second set of beads having a plurality of a second polynucleotide-specific capture primer attached thereon,
 - (iv) a plurality of a first polynucleotide-specific solution-phase primer,
 - (v) a plurality of a second polynucleotide-specific solution phase primer;and
 - b) subjecting the single reaction mixture to an isothermal nucleic acid amplification condition, thereby generating from the first set of beads at least one bead attached to a first substantially monoclonal polynucleotide population containing a first portion of one of the target polynucleotides from the plurality of target polynucleotides, and generating from the second set of beads at least one bead attached to a second substantially monoclonal polynucleotide population containing a second portion of one of the target polynucleotides from the plurality of target polynucleotides.
2. The method of claim 1, wherein the plurality of target polynucleotides is isolated from a biological fluid, cell culture, or solid tissue.
3. The method of claim 2, wherein the plurality of target polynucleotides is isolated from a biological fluid which comprises blood, serum, plasma, saliva, sputum, sweat, tears, lavage fluid, amniotic fluid, cerebrospinal fluid, ascites, urine, or semen.

4. The method of claim 1, wherein the plurality of target polynucleotides comprises genomic DNA.
5. The method of claim 1, wherein the first polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a first target polynucleotide of the plurality of target polynucleotides, and wherein the second polynucleotide-specific capture primer is capable of hybridizing with at least a portion of a second target polynucleotide of the plurality of target polynucleotides.
6. The method of claim 1, wherein the first polynucleotide-specific capture primer further comprises at least one universal sequence that is capable of hybridizing with an amplification primer and/or sequencing primer, and wherein the second polynucleotide-specific capture primer further comprises at least one universal sequence that is capable of hybridizing with an amplification primer and/or sequencing primer.
7. The method of claim 1, wherein the first polynucleotide-specific capture primer comprises at least one unique identifier sequence, and wherein the second polynucleotide-specific capture primer comprises at least one unique identifier sequence.
8. The method of claim 1, wherein the plurality of target polynucleotides contains at least a first target polynucleotide and a second target polynucleotide having different sequences.
9. The method of claim 1, wherein the first polynucleotide-specific solution-phase primer is capable of hybridizing with at least a portion of the plurality of target polynucleotides and/or wherein the second polynucleotide-specific solution-phase primer is capable of hybridizing with at least a portion of the plurality of target polynucleotides.
10. The method of claim 1, wherein the first polynucleotide-specific solution-phase primer comprises at least one universal sequence which is capable of hybridizing with an amplification primer and/or sequencing primer and the first polynucleotide-specific solution-phase primer optionally includes at least one unique identifier sequence, and wherein the second polynucleotide-specific solution-phase primer comprises at least one universal sequence which is capable of hybridizing with an amplification primer and/or

sequencing primer and the second polynucleotide-specific solution-phase primer optionally includes at least one unique identifier sequence.

11. The method of claim 1, wherein the first polynucleotide-specific capture primer and the first polynucleotide-specific solution-phase primer hybridize to different portions of the same target polynucleotide from the plurality of target polynucleotides.
12. The method of claim 11, wherein the portion of the target polynucleotide to which the first polynucleotide-specific capture primer hybridizes is on a first strand of the target polynucleotide in its double-stranded form, and the portion of the target polynucleotide to which the first polynucleotide-specific solution-phase primer hybridizes is on a second strand of the target polynucleotide in its double-stranded form.
13. The method of claim 1, wherein the second polynucleotide-specific capture primer and the second polynucleotide-specific solution-phase primer hybridize to different portions of the same target polynucleotide.
14. The method of claim 13, wherein the portion of the target polynucleotide to which the second polynucleotide-specific capture primer hybridizes is on a first strand of the target polynucleotide in its double-stranded form, and the portion of the target polynucleotide to which the second polynucleotide-specific solution-phase primer hybridizes is on a second strand of the target polynucleotide in its double-stranded form.
15. The method of claim 1, wherein the single reaction mixture comprises a polymerase and a plurality of nucleotides.
16. The method of claim 1, wherein the single reaction mixture comprises a recombinase.
17. The method of claim 16, wherein the recombinase is uvsX.
18. The method of claim 1, wherein the single reaction mixture comprises at least one recombinase loading protein and/or at least one single-stranded binding protein.
19. The method of claim 18, wherein at least one recombinase loading protein is uvsY.

20. The method of claim 18, wherein at least one single-stranded binding protein is gp32.
21. The method of claim 1, further comprising sequencing the first substantially monoclonal polynucleotide population and the second substantially monoclonal polynucleotide population.
22. The method of claim 1, further comprising: depositing onto a first reaction chamber of a support the at least one bead which is attached to the first substantially monoclonal polynucleotide population, and depositing onto a second reaction chamber of the same support the at least one bead which is attached to the second substantially monoclonal polynucleotide population, wherein the support contains an array of reaction chambers which includes the first and second reaction chambers.
23. The method of claim 22, wherein individual reaction chambers in the array, including the first and second reaction chambers, are operatively coupled to at least one sensor that detects a change in the level of hydrogen ions, protons, or phosphate groups.
24. The method of claim 23, further comprising: sequencing the first substantially monoclonal polynucleotide population at the first reaction chamber, and sequencing the second substantially monoclonal polynucleotide population at the second reaction chamber.
25. The method of claim 24, wherein the sequencing comprises detecting a nucleotide incorporation byproduct using the at least one sensor which is operatively coupled to the first reaction chamber, and wherein sequencing comprises detecting a nucleotide incorporation byproduct using the at least one sensor which is operatively coupled to the second reaction chamber.
26. A first set of beads wherein at least one bead is attached to a first substantially monoclonal polynucleotide population containing a first portion of one of the target polynucleotides, and a second set of beads wherein at least one beads is attached to a second substantially monoclonal polynucleotide population containing a second portion

of one of the target polynucleotides, wherein the first and second set of beads are prepared by the method of claim 1.

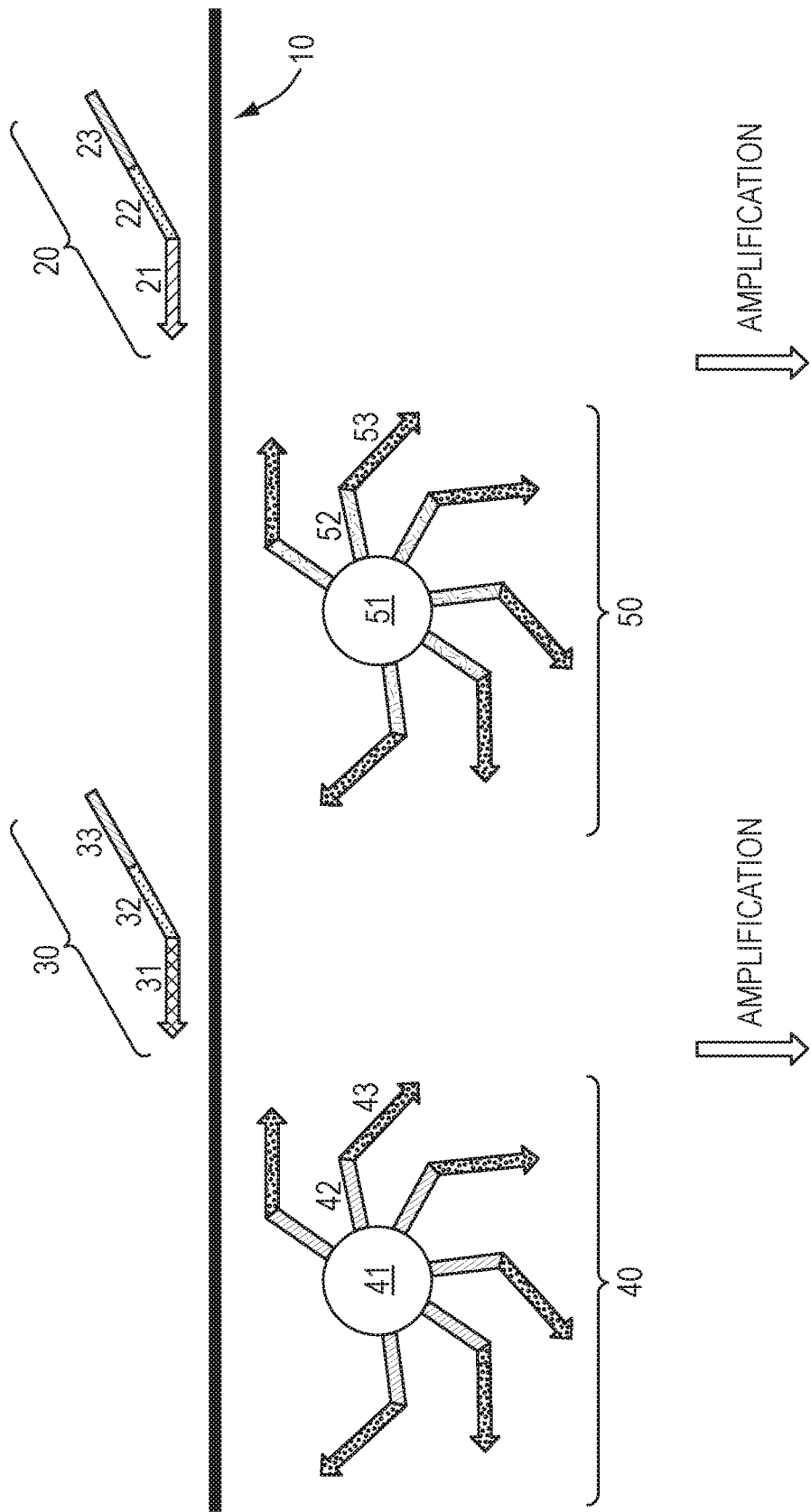


FIG. 1A

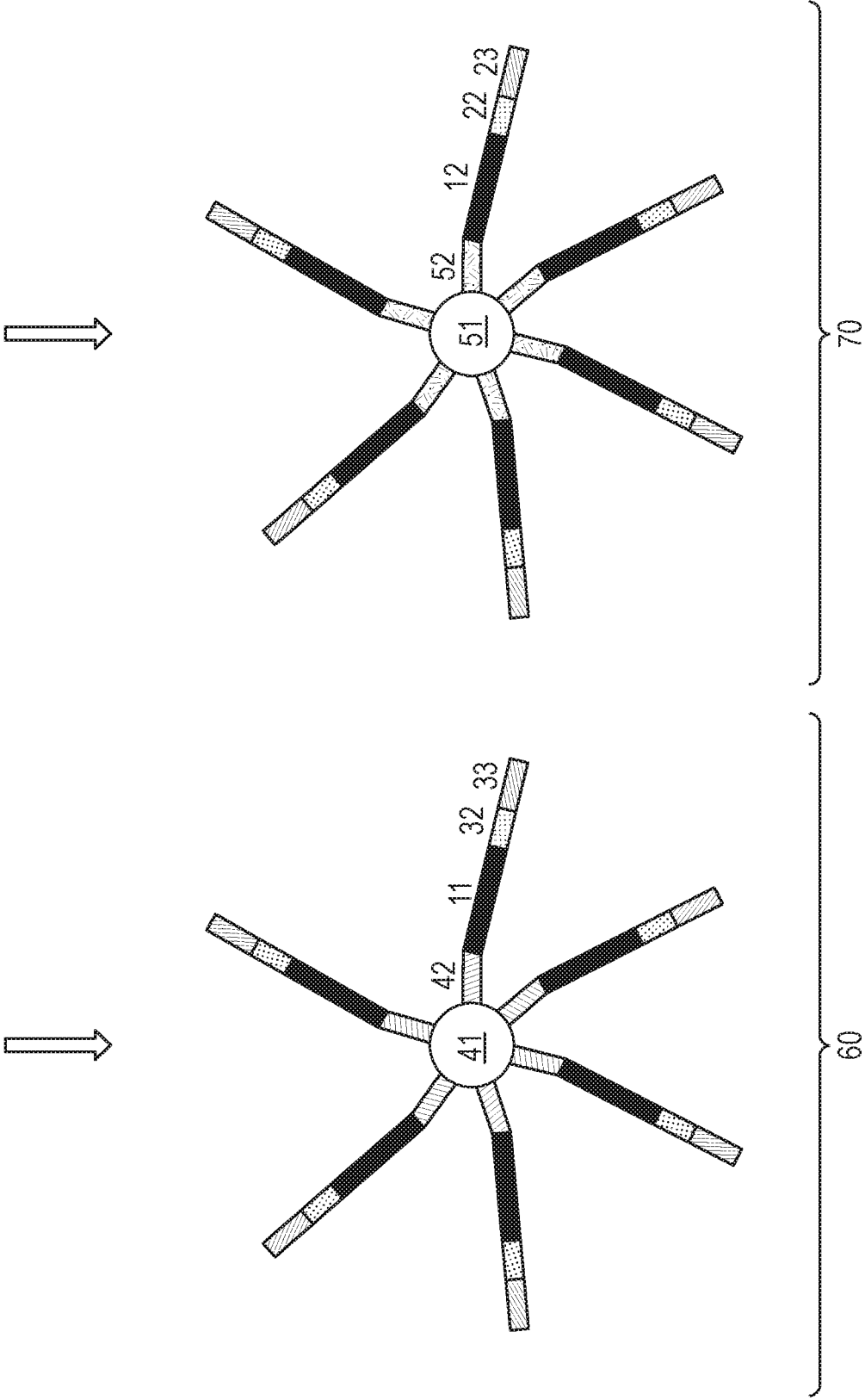


FIG. 1B

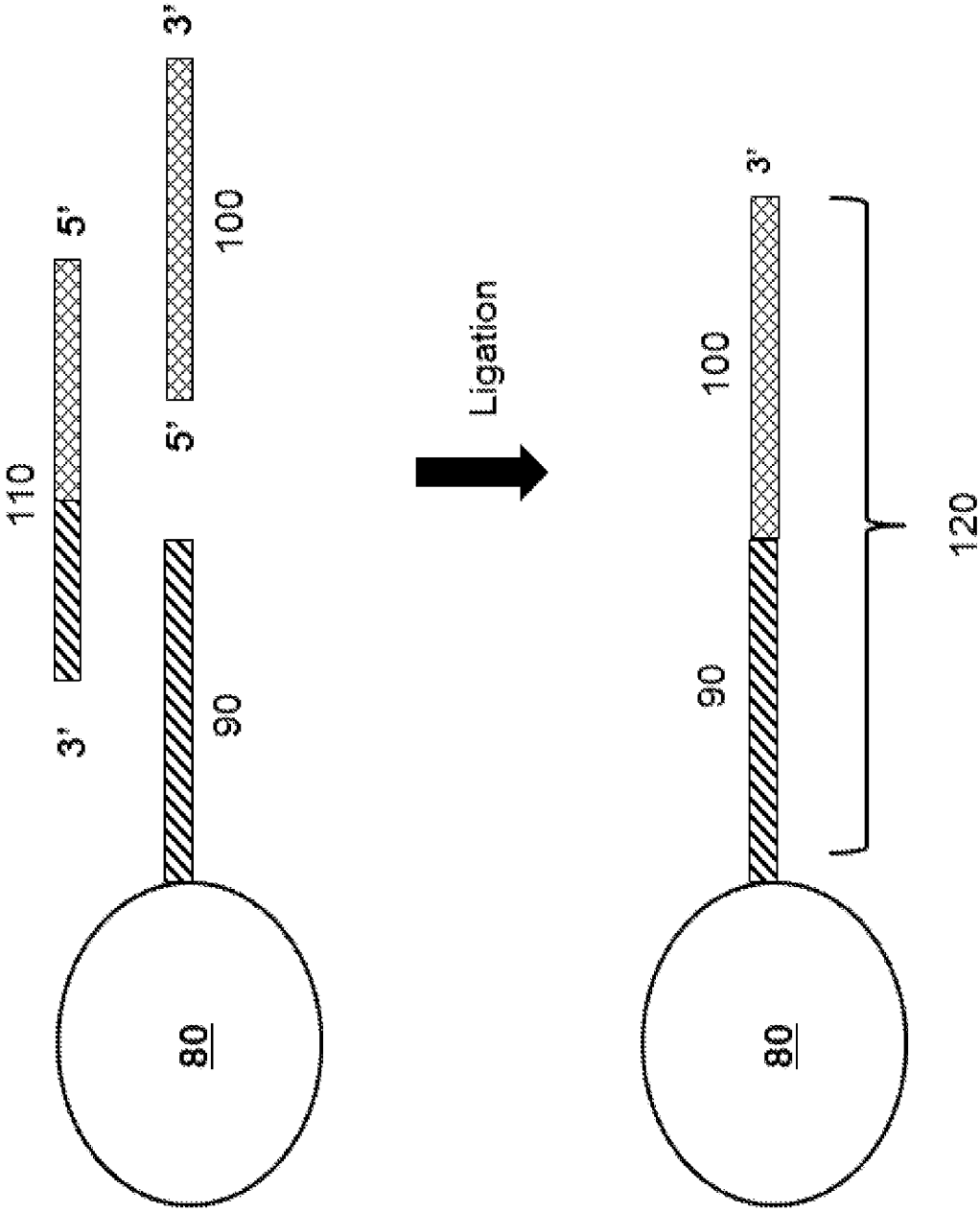


FIG. 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/056113

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 C12Q1/6853 C12Q1/6865
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/191815 A1 (LIFE TECHNOLOGIES CORP [US]) 17 December 2015 (2015-12-17) the whole document	1-26
X,P	WO 2017/161306 A1 (LIFE TECHNOLOGIES CORP [US]) 21 September 2017 (2017-09-21) the whole document	1-26
X	WO 2011/014811 A1 (IBIS BIOSCIENCES INC [US]; HAYDEN MARK A [US]) 3 February 2011 (2011-02-03) the whole document	1-26
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Further documents are listed in the continuation of Box C.



See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

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INTERNATIONAL SEARCH REPORT

International application No

PCT/US2017/056113

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 2015/106941 A1 (ILLUMINA CAMBRIDGE LTD [GB]) 23 July 2015 (2015-07-23) the whole document -----	1-26
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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