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(54) Title: NEXT GENERATION SEQUENCING METHODS

(57) Abstract: The present disclosure provides methods, systems, kits, and compositions for performing sequencing reactions with limiting reagent concentrations. In particular, the present disclosure provides methods of performing next generation sequencing reactions without quantifying sequencing templates (e.g., nucleic acid libraries).

NEXT GENERATION SEQUENCING METHODS

FIELD OF THE DISCLOSURE

The present disclosure provides methods, systems, kits, and compositions for performing sequencing reactions with limiting reagent concentrations. In particular, the present disclosure provides methods of performing next generation sequencing reactions without quantifying sequencing templates (e.g., nucleic acid libraries).

BACKGROUND

Currently libraries must be quantitated using quantitative polymerase chain reaction (qPCR), UV-spectrometry or other assays prior to sequencing to ensure a productive sequencing reaction. In general this causes an increase in time, labor and cost associated with the sequencing process and, in terms of workflow integration (such as with a microfluidic device), also significantly increases the complexity of the device needed to perform the library integration/preparation.

Even in applications not limited by input DNA amounts, library quantification is a substantial source for uncertainty in sequencing outcome: a too high DNA-to-bead ratio leads to a significant fraction of non-readable reactions with multiple DNA templates, while a too low ratio will result in an inadequate amount of reaction and cannot take advantage of the full sequencing capacity.

Methods of performing sequencing reactions without library quantification are needed.

SUMMARY OF THE DISCLOSURE

The present disclosure provides methods, systems, kits, and compositions for performing sequencing reactions with limiting reagent concentrations. In particular, the present disclosure provides methods of performing next generation sequencing reactions without quantifying sequencing templates (e.g., nucleic acid libraries).

For example, in some embodiments, the present disclosure provides a method of sequencing a nucleic acid library where the concentration of the library is unknown, comprising: a) contacting a nucleic acid library that has not been quantitated with a plurality of sequencing reaction components, wherein at least one of the reaction components (e.g., polymerase, dNTPs, or sequencing primers) is present at a limiting concentration; and b) performing the sequencing reaction. In

some embodiments, the sequencing is next generation sequencing (e.g., single molecule sequencing). In some embodiments, the sequencing reaction is performed on a solid support comprising a plurality of reaction sites. In some embodiments, at least one of the reaction components is present at a concentration of one molecule of reaction component per reaction site. In some embodiments, the nucleic acid library is prepared by whole genome amplification. In some embodiments, the whole genome amplification is performed on genomic DNA with at least one amplification reagent present in limiting quantities, and wherein the concentration of the genomic DNA is unknown. In some embodiments, the nucleic acid library has or is suspected of having at least 10,000 (e.g., at least 100,000, at least 1,000,000 or more) target sequences.

Further embodiments provide a method of sequencing a nucleic acid library, comprising: a) contacting a nucleic acid library with a plurality of sequencing reaction components, wherein at least one of said reaction components is present at a molar concentration less than the number of nucleic acid targets in the nucleic acid library; and b) performing the sequencing reaction. In some embodiments, the concentration of nucleic acids in the nucleic acid library is not quantitated.

Additional embodiments provide a system comprising: a) a container consisting of, consisting essentially of, or comprising a nucleic acid sequencing library that has not been quantitated and a plurality of reaction components, wherein at least one of the reaction components is present at limiting concentration; and b) a nucleic acid sequencing device or system.

Other embodiments provide a system comprising: a) a container consisting of, consisting essentially of, or comprising a first number of molecules of sequencing library nucleic acid targets and a second number of polymerase molecules, wherein the second number of molecules is less than the first number of molecules (e.g., 2 times, 4 time, 10 times, 100 times less or lower); and b) a nucleic acid sequencing device or system.

Additional embodiments are described herein.

DESCRIPTION OF THE FIGURES

Figure 1 shows a schematic of a prior art preparation where a DNA library is quantitated prior to sequencing, and none of the reagents in the sequencing reaction are provided in a limiting amount.

Figure 2 shows an exemplary embodiment of the present disclosure where no DNA library quantitation is conducted (and no dilution series are prepared), and a specific limiting amount of a sequencing component is provided (e.g., polymerase in this figure) such that only productive sequencing complexes are formed prior to sequencing.

Figure 3 shows the number of sequencing reads versus library concentration.

Figure 4 shows normalization of input into library preparation process by limitation of a key reaction component. As described in Example 2, a 1,000,000 fold difference in input amount (100 ng to 100 fg) was normalized to within 5 fold output (10 μg to 2 μg) by limiting the amount of dNTPs used in the reaction. This allows a large range of input concentrations to be successfully used with the library preparation process without the need for quantification. Changing the dNTP concentration and time allows for adjustment of the output amount created.

DEFINITIONS

As used herein, the term "amplifying" or "amplification" in the context of nucleic acids refers to the production of multiple copies of a polynucleotide, or a portion of the polynucleotide, typically starting from a small amount of the polynucleotide (e.g., a single polynucleotide molecule), where the amplification products or amplicons are generally detectable. Amplification of polynucleotides encompasses a variety of chemical and enzymatic processes. The generation of multiple DNA copies from one or a few copies of a target or template DNA molecule during a polymerase chain reaction (PCR) or a ligase chain reaction (LCR) are forms of amplification. Amplification is not limited to the strict duplication of the starting molecule. For example, the generation of multiple cDNA molecules from a limited amount of RNA in a sample using reverse transcription (RT)-PCR is a form of amplification. Furthermore, the generation of multiple RNA molecules from a single DNA molecule during the process of transcription is also a form of amplification.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, that is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product that is complementary to a nucleic acid strand is induced (*e.g.*, in the presence of nucleotides and an inducing agent such as a biocatalyst (*e.g.*, a DNA polymerase or the like) and at a suitable temperature and

pH). The primer is typically single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is generally first treated to separate its strands before being used to prepare extension products. In some embodiments, the primer is an oligodeoxyribonucleotide. The primer is sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "sample" refers to anything capable of being analyzed by the methods provided herein that is suspected of containing a target nucleic acid sequence. Samples may be complex samples or mixed samples, which contain nucleic acids comprising multiple different nucleic acid sequences. Samples may comprise nucleic acids from more than one source (e.g. difference species, different subspecies, etc.), subject, and/or individual. In some embodiments, the methods provided herein comprise purifying the sample or purifying the nucleic acid(s) from the sample. In some embodiments, the sample contains purified nucleic acid. In some embodiments, a sample is derived from a biological, clinical, environmental, research, forensic, or other source.

DETAILED DESCRIPTION

The present disclosure provides methods, systems, kits, and compositions for performing sequencing reactions with limiting reagent concentrations. In particular, the present disclosure provides methods of performing next generation sequencing reactions without quantifying sequencing templates (e.g., nucleic acid libraries).

The systems and methods of the present disclosure eliminate the need to quantitate sequencing libraries prior to performing next generation sequencing by limiting an element of the sequencing reaction (e.g., polymerase or primers). This is important because currently all next generation sequencing technologies require very accurate quantitation of the sequencing libraries prior to sequencing, which adds significant time and labor to the next generation sequencing process and makes integration of the workflow (e.g., in a microfluidic device) significantly more complex and expensive. Additionally, even after this laborious quantitation process, current technologies still often require further empirical validation (for example a dilution series of library) of this quantitation prior to full scale sequencing.

Embodiments of the present disclosure avoid the need to quantitate sequencing templates (e.g., libraries) prior to sequencing by limiting one or more reagents of the sequencing reaction (e.g., nucleic acid polymerase, sequencing primers, nucleotides, etc.). This controls the number of molecules that are being sequenced and assures that there are an optima and known number or transcripts being sequenced.

The methods of the present disclosure achieve the same sequencing performance using less time, less labor and at a lower cost. Additionally, the complexity of the device/instrumentation needed to perform the integration of the sequencing workflow is significantly reduced.

The present disclosure finds use in a variety of sequencing techniques (e.g., next generation sequencing). In some embodiments, the sequencing nucleic acid library has or is suspected of having at least 10,000 (e.g., at least 100,000, at least 1,000,000 or more) target sequences. In some embodiments, sequencing methods are single molecule sequencing methods. For example, in single molecule sequencing methods, it is optimal to have one molecule of DNA per limiting reagent (e.g., polymerase). In embodiments of the present disclosure, instead of quantitating the input library, the amount of polymerase or other reagent is limited so that there is less reagent than DNA. For example, in some embodiments, the amount of polymerase is optimized such that one molecule of polymerase or primer per reaction site (e.g., on a solid support) is provided.

In some embodiments, the sequencing is the real-time single molecule sequencing system developed by Pacific Biosciences (Voelkerding *et al.*, *Clinical Chem.*, *55:* 641-658, 2009; MacLean *et al.*, *Nature Rev. Microbiol.*, *7:* 287-296; U.S. Pat. No. 7,170,050; U.S. Pat. No. 7,302,146; U.S. Pat. No. 7,313,308; U.S. Pat. No. 7,476,503; all of which are herein incorporated by reference) utilizes reaction wells 50-100 nm in diameter and encompassing a reaction volume of approximately 20 zeptoliters (10×10^{-21} L). Sequencing reactions are performed using immobilized template, modified phi29 DNA polymerase, and high local concentrations of fluorescently labeled dNTPs. High local concentrations and continuous reaction conditions allow incorporation events to be captured in real time by fluor signal detection using laser excitation, an optical waveguide, and a CCD camera.

In certain embodiments, the single molecule real time (SMRT) DNA sequencing methods using zero-mode waveguides (ZMWs) developed by Pacific Biosciences, or similar methods, are employed. With this technology, DNA

sequencing is performed on SMRT chips, each containing thousands of zero-mode waveguides (ZMWs). A ZMW is a hole, tens of nanometers in diameter, fabricated in a 100nm metal film deposited on a silicon dioxide substrate. Each ZMW becomes a nanophotonic visualization chamber providing a detection volume of just 20 zeptoliters (10-21 liters). At this volume, the activity of a single molecule can be detected amongst a background of thousands of labeled nucleotides.

The ZMW provides a window for watching DNA polymerase as it performs sequencing by synthesis. Within each chamber, a single DNA polymerase molecule is attached to the bottom surface such that it permanently resides within the detection volume. Phospholinked nucleotides, each type labeled with a different colored fluorophore, are then introduced into the reaction solution at high concentrations which promote enzyme speed, accuracy, and processivity. Due to the small size of the ZMW, even at these high, biologically relevant concentrations, the detection volume is occupied by nucleotides only a small fraction of the time. In addition, visits to the detection volume are fast, lasting only a few microseconds, due to the very small distance that diffusion has to carry the nucleotides. The result is a very low background.

Processes and systems for such real time sequencing that may be adapted for use with the disclosure are described in, for example, U.S. Patent Nos. 7,405,281, entitled "Fluorescent nucleotide analogs and uses therefor;" 7,315,019, entitled "Arrays of optical confinements and uses thereof;' 7,313,308, entitled "Optical analysis of molecules," 7,302,146, entitled "Apparatus and method for analysis of molecules" , and 7,170,050,
entitled "Apparatus and methods for optical analysis of molecules," U.S. Patent Publications Nos. 20080212960, entitled "Methods and systems for simultaneous real-time monitoring of optical signals from multiple sources", 20080206764, entitled "Flowcell system for single molecule detection", 20080199932, entitled "Active surface coupled polymerases", 20080199874, entitled "CONTROLLABLE STRAND SCISSION OF MINI CIRCLE DNA", 20080176769, entitled "Articles having localized molecules disposed thereon and methods of producing same", 20080176316, entitled "Mitigation of photodamage in analytical reactions", 20080176241, entitled "Mitigation of photodamage in analytical reactions", 20080165346, entitled "Methods and systems for simultaneous real-time monitoring of optical signals from multiple sources", 20080160531, entitled "Uniform surfaces for hybrid material substrates and

methods for making and using same", 20080157005, entitled "Methods and systems for simultaneous real-time monitoring of optical signals from multiple sources", 20080153100, entitled "Articles having localized molecules disposed thereon and methods of producing same", 20080153095, entitled "CHARGE SWITCH NUCLEOTIDES", 20080152281, entitled "Substrates, systems and methods for analyzing materials", 20080152280, entitled "Substrates, systems and methods for analyzing materials", 20080145278, entitled "Uniform surfaces for hybrid material substrates and methods for making and using same", 20080128627, entitled "SUBSTRATES, SYSTEMS AND METHODS FOR ANALYZING MATERIALS", 20080108082, entitled "Polymerase enzymes and reagents for enhanced nucleic acid sequencing", 20080095488, entitled "SUBSTRATES FOR PERFORMING ANALYTICAL REACTIONS", 20080080059, entitled "MODULAR OPTICAL COMPONENTS AND SYSTEMS INCORPORATING SAME", 20080050747, entitled "Articles having localized molecules disposed thereon and methods of producing and using same", 20080032301, entitled "Articles having localized molecules disposed thereon and methods of producing same", 20080030628, entitled "Methods and systems for simultaneous real-time monitoring of optical signals from multiple sources", 20080009007, entitled "CONTROLLED INITIATION OF PRIMER EXTENSION", 20070238679, entitled "Articles having localized molecules disposed thereon and methods of producing same", 20070231804, entitled "Methods, systems and compositions for monitoring enzyme activity and applications thereof', 20070206187, entitled "Methods and systems for simultaneous real-time monitoring of optical signals from multiple sources", 20070196846, entitled "Polymerases for nucleotide analogue incorporation", 20070188750, entitled "Methods and systems for simultaneous real-time monitoring of optical signals from multiple sources", 20070161017, entitled "MITIGATION OF PHOTODAMAGE IN ANALYTICAL REACTIONS", 20070141598, entitled "Nucleotide Compositions and Uses Thereof', 20070134128, entitled "Uniform surfaces for hybrid material substrate and methods for making and using same", 20070128133, entitled "Mitigation of photodamage in analytical reactions", 20070077564, entitled "Reactive surfaces, substrates and methods of producing same", 20070072196, entitled "Fluorescent nucleotide analogs and uses therefore", and 20070036511, entitled "Methods and systems for monitoring multiple optical signals from a single source", and Korlach et al. (2008) "Selective aluminum passivation for targeted immobilization of single DNA polymerase

molecules in zero-mode waveguide nanostructures" Proc. Nat'I. Acad. Sci. U.S.A. 105(4): 11761181 - all of which are herein incorporated by reference in their entireties.

A number of other DNA sequencing techniques can be used, including fluorescence-based sequencing methodologies (See, e.g., Birren et al., Genome Analysis: Analyzing DNA, 1, Cold Spring Harbor, N.Y.; herein incorporated by reference in its entirety). In some embodiments, automated sequencing techniques understood in that art are utilized. In some embodiments, DNA sequencing is achieved by parallel oligonucleotide extension (See, e.g., U.S. Pat. No. 5,750,341 to Macevicz et al., and U.S. Pat. No. 6,306,597 to Macevicz et al., both of which are herein incorporated by reference in their entireties). Additional examples of sequencing techniques include the Church polony technology (Mitra et al., 2003, Analytical Biochemistry 320, 55-65; Shendure et al., 2005 Science 309, 1728-1732; U.S. Pat. No. 6,432,360, U.S. Pat. No. 6,485,944, U.S. Pat. No. 6,511,803; herein incorporated by reference in their entireties) the 454 picotiter pyrosequencing technology (Margulies et al., 2005 Nature 437, 376-380; US 20050130173; herein incorporated by reference in their entireties), the Solexa single base addition technology (Bennett et al., 2005, Pharmacogenomics, 6, 373-382; U.S. Pat. No. 6,787,308; U.S. Pat. No. 6,833,246; herein incorporated by reference in their entireties), the Lynx massively parallel signature sequencing technology (Brenner et al. (2000). Nat. Biotechnol. 18:630-634; U.S. Pat. No. 5,695,934; U.S. Pat. No. 5,714,330; herein incorporated by reference in their entireties) and the Adessi PCR colony technology (Adessi et al. (2000). Nucleic Acid Res. 28, E87; WO 00018957; herein incorporated by reference in its entirety).

In some embodiments, chain terminator sequencing is utilized. Chain terminator sequencing uses sequence-specific termination of a DNA synthesis reaction using modified nucleotide substrates. Extension is initiated at a specific site on the template DNA by using a short radioactive, or other labeled, oligonucleotide primer complementary to the template at that region. The oligonucleotide primer is extended using a DNA polymerase, standard four deoxynucleotide bases, and a low concentration of one chain terminating nucleotide, most commonly a di-deoxynucleotide. This reaction is repeated in four separate tubes with each of the bases taking turns as the di-deoxynucleotide. Limited incorporation of the chain terminating nucleotide by the DNA polymerase results in a series of related DNA

fragments that are terminated only at positions where that particular di-deoxynucleotide is used. For each reaction tube, the fragments are size-separated by electrophoresis in a slab polyacrylamide gel or a capillary tube filled with a viscous polymer. The sequence is determined by reading which lane produces a visualized mark from the labeled primer as you scan from the top of the gel to the bottom.

Dye terminator sequencing alternatively labels the terminators. Complete sequencing can be performed in a single reaction by labeling each of the di-deoxynucleotide chain-terminators with a separate fluorescent dye, which fluoresces at a different wavelength.

A set of methods referred to as "next-generation sequencing" techniques have emerged as alternatives to Sanger and dye-terminator sequencing methods (Voelkerding *et al.*, *Clinical Chem.*, *55:* 641-658, 2009; MacLean *et al.*, *Nature Rev. Microbiol.*, *7:* 287-296; each herein incorporated by reference in their entirety). Next-generation sequencing (NGS) methods share the common feature of massively parallel, high-throughput strategies, with the goal of lower costs in comparison to older sequencing methods. NGS methods can be broadly divided into those that require template amplification and those that do not. Amplification-requiring methods include pyrosequencing commercialized by Roche as the 454 technology platforms (e.g., GS 20 and GS FLX), the Solexa platform commercialized by Illumina, and the Supported Oligonucleotide Ligation and Detection (SOLiD) platform commercialized by Applied Biosystems. Non-amplification approaches, also known as single-molecule sequencing, are exemplified by the HeliScope platform commercialized by Helicos BioSciences, and emerging platforms commercialized by VisiGen, Oxford Nanopore Technologies Ltd., and Pacific Biosciences, respectively.

In pyrosequencing (Voelkerding *et al.*, *Clinical Chem.*, *55:* 641-658, 2009; MacLean *et al.*, *Nature Rev. Microbiol.*, *7:* 287-296; U.S. Pat. No. 6,210,891; U.S. Pat. No. 6,258,568; each herein incorporated by reference in its entirety), template DNA is fragmented, end-repaired, ligated to adaptors, and clonally amplified in-situ by capturing single template molecules with beads bearing oligonucleotides complementary to the adaptors. Each bead bearing a single template type is compartmentalized into a water-in-oil microvesicle, and the template is clonally amplified using a technique referred to as emulsion PCR. The emulsion is disrupted after amplification and beads are deposited into individual wells of a picotitre plate

functioning as a flow cell during the sequencing reactions. Ordered, iterative introduction of each of the four dNTP reagents occurs in the flow cell in the presence of sequencing enzymes and luminescent reporter such as luciferase. In the event that an appropriate dNTP is added to the 3' end of the sequencing primer, the resulting production of ATP causes a burst of luminescence within the well, which is recorded using a CCD camera. It is possible to achieve read lengths greater than or equal to 400 bases, and 1 x 10^6 sequence reads can be achieved, resulting in up to 500 million base pairs (Mb) of sequence.

In the Solexa/Illumina platform (Voelkerding *et al.*, *Clinical Chem.*, *55:* 641-658, 2009; MacLean *et al.*, *Nature Rev. Microbiol.*, *7:* 287-296; U.S. Pat. No. 6,833,246; U.S. Pat. No. 7,115,400; U.S. Pat. No. 6,969,488; each herein incorporated by reference in its entirety), sequencing data are produced in the form of shorter-length reads. In this method, single-stranded fragmented DNA is end-repaired to generate 5'-phosphorylated blunt ends, followed by Klenow-mediated addition of a single A base to the 3' end of the fragments. A-addition facilitates addition of T-overhang adaptor oligonucleotides, which are subsequently used to capture the template-adaptor molecules on the surface of a flow cell that is studded with oligonucleotide anchors. The anchor is used as a PCR primer, but because of the length of the template and its proximity to other nearby anchor oligonucleotides, extension by PCR results in the "arching over" of the molecule to hybridize with an adjacent anchor oligonucleotide to form a bridge structure on the surface of the flow cell. These loops of DNA are denatured and cleaved. Forward strands are then sequenced with reversible dye terminators. The sequence of incorporated nucleotides is determined by detection of post-incorporation fluorescence, with each fluor and block removed prior to the next cycle of dNTP addition. Sequence read length ranges from 36 nucleotides to over 50 nucleotides, with overall output exceeding 1 billion nucleotide pairs per analytical run.

Sequencing nucleic acid molecules using SOLiD technology (Voelkerding *et al.*, *Clinical Chem.*, *55:* 641-658, 2009; MacLean *et al.*, *Nature Rev. Microbiol.*, *7:* 287-296; U.S. Pat. No. 5,912,148; U.S. Pat. No. 6,130,073; each herein incorporated by reference in their entirety) also involves fragmentation of the template, ligation to oligonucleotide adaptors, attachment to beads, and clonal amplification by emulsion PCR. Following this, beads bearing template are immobilized on a derivatized surface of a glass flow-cell, and a primer complementary to the adaptor

oligonucleotide is annealed. However, rather than utilizing this primer for 3' extension, it is instead used to provide a 5' phosphate group for ligation to interrogation probes containing two probe-specific bases followed by 6 degenerate bases and one of four fluorescent labels. In the SOLiD system, interrogation probes have 16 possible combinations of the two bases at the 3' end of each probe, and one of four fluors at the 5' end. Fluor color and thus identity of each probe corresponds to specified color-space coding schemes. Multiple rounds (usually 7) of probe annealing, ligation, and fluor detection are followed by denaturation, and then a second round of sequencing using a primer that is offset by one base relative to the initial primer. In this manner, the template sequence can be computationally re-constructed, and template bases are interrogated twice, resulting in increased accuracy. Sequence read length averages 35 nucleotides, and overall output exceeds 4 billion bases per sequencing run.

In certain embodiments, nanopore sequencing in employed (see, e.g., Astier et al., J Am Chem Soc. 2006 Feb 8;128(5):1705-10, herein incorporated by reference). The theory behind nanopore sequencing has to do with what occurs when the nanopore is immersed in a conducting fluid and a potential (voltage) is applied across it: under these conditions a slight electric current due to conduction of ions through the nanopore can be observed, and the amount of current is exceedingly sensitive to the size of the nanopore. If DNA molecules pass (or part of the DNA molecule passes) through the nanopore, this can create a change in the magnitude of the current through the nanopore, thereby allowing the sequences of the DNA molecule to be determined.

In certain embodiments, HeliScope by Helicos BioSciences is employed (Voelkerding *et al.*, *Clinical Chem.*, *55:* 641-658, 2009; MacLean *et al.*, *Nature Rev. Microbiol.*, *7:* 287-296; U.S. Pat. No. 7,169,560; U.S. Pat. No. 7,282,337; U.S. Pat. No. 7,482,120; U.S. Pat. No. 7,501,245; U.S. Pat. No. 6,818,395; U.S. Pat. No. 6,911,345; U.S. Pat. No. 7,501,245; each herein incorporated by reference in their entirety). Template DNA is fragmented and polyadenylated at the 3' end, with the final adenosine bearing a fluorescent label. Denatured polyadenylated template fragments are ligated to poly(dT) oligonucleotides on the surface of a flow cell. Initial physical locations of captured template molecules are recorded by a CCD camera, and then label is cleaved and washed away. Sequencing is achieved by addition of polymerase and serial addition of fluorescently-labeled dNTP reagents.

Incorporation events result in fluor signal corresponding to the dNTP, and signal is captured by a CCD camera before each round of dNTP addition. Sequence read length ranges from 25-50 nucleotides, with overall output exceeding 1 billion nucleotide pairs per analytical run.

In certain embodiments, the Ion Torrent technology (Life Technologies) is employed to sequence purified target nucleic acid sequences. The Ion Torrent technology is a method of DNA sequencing based on the detection of hydrogen ions that are released during the polymerization of DNA (see, e.g., *Science* **327**(5970): 1190 (2010); U.S. Pat. Appl. Pub. Nos. 20090026082, 20090127589, 20100301398, 20100197507, 20100188073, and 20100137143, incorporated by reference in their entireties for all purposes). A microwell contains a template DNA strand to be sequenced. Beneath the layer of microwells is a hypersensitive ISFET ion sensor. All layers are contained within a CMOS semiconductor chip, similar to that used in the electronics industry. When a dNTP is incorporated into the growing complementary strand a hydrogen ion is released, which triggers a hypersensitive ion sensor. If homopolymer repeats are present in the template sequence, multiple dNTP molecules will be incorporated in a single cycle. This leads to a corresponding number of released hydrogens and a proportionally higher electronic signal. This technology differs from other sequencing technologies in that no modified nucleotides or optics are used. The per-base accuracy of the Ion Torrent sequencer is ~99.6% for 50 base reads, with ~100 Mb generated per run. The read-length is 100 base pairs. The accuracy for homopolymer repeats of 5 repeats in length is ~98%. The benefits of ion semiconductor sequencing are rapid sequencing speed and low upfront and operating costs.

Another exemplary nucleic acid sequencing approach that may be adapted for use with the present disclosure was developed by Stratos Genomics, Inc. and involves the use of Xpandomers. This sequencing process typically includes providing a daughter strand produced by a template-directed synthesis. The daughter strand generally includes a plurality of subunits coupled in a sequence corresponding to a contiguous nucleotide sequence of all or a portion of a target nucleic acid in which the individual subunits comprise a tether, at least one probe or nucleobase residue, and at least one selectively cleavable bond. The selectively cleavable bond(s) is/are cleaved to yield an Xpandomer of a length longer than the plurality of the subunits of the daughter strand. The Xpandomer typically includes the tethers and reporter elements

for parsing genetic information in a sequence corresponding to the contiguous nucleotide sequence of all or a portion of the target nucleic acid. Reporter elements of the Xpandomer are then detected. Additional details relating to Xpandomer-based approaches are described in, for example, U.S. Patent Publication No. 20090035777.

Other emerging single molecule sequencing methods include real-time sequencing by synthesis using a VisiGen platform (Voelkerding *et al.*, *Clinical Chem.*, *55:* 641-658, 2009; U.S. Pat. No. 7,329,492; U.S. Pat. App. Ser. No. 11/671956; U.S. Pat. App. Ser. No. 11/ 781166; each herein incorporated by reference in their entirety) in which immobilized, primed DNA template is subjected to strand extension using a fluorescently-modified polymerase and florescent acceptor molecules, resulting in detectible fluorescence resonance energy transfer (FRET) upon nucleotide addition.

In certain embodiments, the target nucleic acid sequences (e.g., genomic DNA) are subjected to amplification (e.g., prior to sequencing or during library preparation). In some embodiments, amplification is whole genome amplification.

Exemplary amplification reactions include, but are not limited to the polymerase chain reaction (PCR) or ligase chain reaction (LCR), each of which is driven by thermal cycling. Amplifications used in method or assays of the present disclosure may be performed in bulk and/or partitioned volumes (e.g. droplets). Alternative amplification reactions, which may be performed isothermally, also find use herein, such as branched-probe DNA assays, cascade-RCA, helicase-dependent amplification, loop-mediated isothermal amplification (LAMP), nucleic acid based amplification (NASBA), nicking enzyme amplification reaction (NEAR), PAN-AC, Q-beta replicase amplification, rolling circle replication (RCA), self-sustaining sequence replication, strand-displacement amplification, and the like.

Amplification may be performed with any suitable reagents (e.g. template nucleic acid (e.g. DNA or RNA), primers, probes, buffers, replication catalyzing enzyme (e.g. DNA polymerase, RNA polymerase), nucleotides, salts (e.g. $MgCl_2$), etc. In some embodiments, an amplification mixture includes any combination of at least one primer or primer pair, at least one probe, at least one replication enzyme (e.g., at least one polymerase, such as at least one DNA and/or RNA polymerase), and deoxynucleotide (and/or nucleotide) triphosphates (dNTPs and/or NTPs), etc.

In some embodiments, the present disclosure utilizes nucleic acid amplification that relies on alternating cycles of heating and cooling (i.e., thermal

cycling) to achieve successive rounds of replication (e.g., PCR). In some embodiments, PCR is used to amplify target nucleic acids (e.g. partitioned targets). PCR may be performed by thermal cycling between two or more temperature set points, such as a higher melting (denaturation) temperature and a lower annealing/extension temperature, or among three or more temperature set points, such as a higher melting temperature, a lower annealing temperature, and an intermediate extension temperature, among others. PCR may be performed with a thermostable polymerase, such as Taq DNA polymerase (e.g., wild-type enzyme, a Stoffel fragment, FastStart polymerase, etc.), Pfu DNA polymerase, S-Tbr polymerase, Tth polymerase, Vent polymerase, or a combination thereof, among others. Typical PCR methods produce an exponential increase in the amount of a product amplicon over successive cycles, although linear PCR methods also find use in the present disclosure.

The methods, compositions, systems, and devices of the present disclosure make use of samples which include, or are suspected of including, a target nucleic acid sequence. Samples may be derived from any suitable source, and for purposes related to any field, including but not limited to diagnostics, research, forensics, epidemiology, pathology, archaeology, etc. A sample may be biological, environmental, forensic, veterinary, clinical, etc. in origin. Samples may include nucleic acid derived from any suitable source, including eukaryotes, prokaryotes (e.g. infectious bacteria), mammals, humans, non-human primates, canines, felines, bovines, equines, porcines, mice, viruses, etc. Samples may contain, e.g., whole organisms, organs, tissues, cells, organelles (e.g., chloroplasts, mitochondria), synthetic nucleic acid, cell lysate, etc. Nucleic acid present in a sample (e.g. target nucleic acid, template nucleic acid, non-target nucleic acid, contaminant nucleic acid may be of any type, e.g., genomic DNA, RNA, plasmids, bacteriophages, synthetic origin, natural origin, and/or artificial sequences (non-naturally occurring), synthetically-produced but naturally occurring sequences, etc. Biological specimens may, for example, include whole blood, lymphatic fluid, serum, plasma, sweat, tear, saliva, sputum, cerebrospinal (CSF) fluids, amniotic fluid, seminal fluid, vaginal excretions, serous fluid, synovial fluid, pericardial fluid, peritoneal fluid, pleural fluid, transudates, exudates, cystic fluid, bile, urine, gastric fluids, intestinal fluids, fecal samples, and swabs or washes (e.g., oral, nasopharangeal, optic, rectal, intestinal, vaginal, epidermal, etc.) and/or other biological specimens.

In some embodiments, samples that find use with the present disclosure are mixed samples (e.g. containing mixed nucleic acid populations). In some embodiments, samples analyzed by methods herein contain, or may contain, a plurality of different nucleic acid sequences. In some embodiments, a sample (e.g. mixed sample) contains one or more nucleic acid molecules (e.g. 1… 10… 10^2… 10^3… 10^4… 10^5… 10^6… 10^7, etc.) that contain a target sequence of interest in a particular application. In some embodiments, a sample (e.g. mixed sample) contains zero nucleic acid molecules that contain a target sequence of interest in a particular application. In some embodiments, a sample (e.g. mixed sample) contains nucleic acid molecules with a plurality of different sequences that all contain a target sequence of interest. In some embodiments, a sample (e.g. mixed sample) contains one or more nucleic acid molecules (e.g. 1… 10… 10^2… 10^3…10^4… 10^5… 10^6… 10^7, etc.) that do not contain a target sequence of interest in a particular application. In some embodiments, a sample (e.g. mixed sample) contains zero nucleic acid molecules that do not contain a target sequence of interest in a particular application. In some embodiments, a sample (e.g. mixed sample) contains nucleic acid molecules with a plurality of different sequences that do not contain a target sequence of interest. In some embodiments, a sample contains more nucleic acid molecules that do not contain a target sequence than nucleic acid molecules that do contain a target sequence (e.g. 1.01:1… 2:1… 5:1… 10:1… 20:1… 50:1… 10^2:1… 10^3:1…10^4:1… 10^5:1… 10^6:1… 10^7:1). In some embodiments, a sample contains more nucleic acid molecules that do contain a target sequence than nucleic acid molecules that do not contain a target sequence (e.g. 1.01:1… 2:1… 5:1… 10:1… 20:1… 50:1… 10^2:1… 10^3:1…10^4:1… 10^5:1… 10^6:1… 10^7:1). In some embodiments, a sample contains a single target sequence which may be present in one or more nucleic acid molecules in the sample. In some embodiments, a sample contains two or more target sequences (e.g. 2, 3, 4, 5…10…20…50…100, etc.) which may each be present in one or more nucleic acid molecules in the sample.

EXAMPLES

Example 1

Sequencing with Limited Polymerase

Methods

Genomic DNA was carried through a library preparation protocol to create structurally linear, topologically circular DNA template for sequencing. In short this procedure: (1) amplified the DNA via a whole genome amplification (WGA) method (2) fragmented the DNA to an appropriate length using ultrasonic energy (3) prepared the fragmented DNA for ligation using a combination of polymerases and a kinase (4) attached sequencing adapters using a ligase (5) removed incomplete products using a combination of exonucleases (6) purified the sequencing library using a magnetic bead based purification method.

This template was then annealed to a sequencing primer which is complementary to a sequence in the ligated sequencing adapters. These annealed complexes were then incubated with various amounts of sequencing polymerase and the reactions sequenced on a Pacific Biosciences RS sequencer.

Results

The three concentrations of library (1x, 5x, 10x) all showed very similar average readlengths and read qualities. The # of sequencing reads was slightly elevated (~50%) at the 5x and 10x library concentrations in comparison to the 1x library concentration. (See Figure 3)

The three metrics discussed above (# sequencing reads, average readlength, and read quality) are the key metrics used to determine the success of a sequencing run. As the read quality and average readlength remained essentially unchanged between the 1x, 5x and 10x library concentrations, it is clear that changes in the library concentration have no effect when a key sequencing component (in this case polymerase) is limited.

For the average # of sequencing reads, normally 5x and 10x the amount of template would result in a large increase in available template/polymerase complex leading to overloading of the sequencing reaction and a large reduction in the # of sequencing reads. However, by limiting a sequencing component (in this case polymerase) the average # of sequencing reads is actually slightly elevated (~50%) when 5x and 10x the normal amount of library is added.

Equal or improved performance at higher library to polymerase ratio indicates ability to load libraries within at least 10 fold concentration range by limiting an element of the sequencing reaction (e.g. polymerase).

Example 2

Sequencing with Library Quantitation

Various amounts of genomic DNA were mixed with: reaction buffer, primers (in this case random septamers), a combination of polymerases (in this case Phi 29 and Klenow exo-), an accessory enzyme (in this case pyrophosphatase) and dNTPs. The reactions were then carried through a temperature cycle that results in amplification of the input DNA (in this case a constant 37° C for two hours.)

At all dNTP concentrations (22 μM to 175 μM) a large range of input concentrations (100 ng to 100 fg) gave outputs with much smaller concentration ranges. Figure 4 shows normalization of input into library preparation process by limitation of a reaction component. A 1,000,000 fold difference in input amount (100 ng to 100 fg) was normalized to within 5 fold output (10 μg to 2 μg) by limiting the amount of dNTPs used in the reaction. This allows a large range of input concentrations to be successfully used with the library preparation process without the need for quantification. Changing the dNTP concentration and time allows for adjustment of the output amount created.

This is just one example of how to use an enzymatic reaction (in this case whole genome amplification using an improved multiple displacement amplification) to normalize a sample's concentration for subsequent sample processing. Without this normalization it would be difficult to develop a downstream process (in this case a next generation sequencing library prep) without including a time consuming and potentially complicated quantitation step. Removal of the need for the quantitation step allows for a simplified workflow and makes it much easier to integrate the workflow into an automated device (such as a microfluidic cartridge).

Although the disclosure has been described in connection with specific preferred embodiments, it should be understood that the disclosure as claimed should not be unduly limited to such specific embodiments. Various modification and variation of the described methods and compositions of the disclosure will be apparent to those skilled in the art without departing from the scope and spirit of the

disclosure. Indeed, various modifications of the described modes for carrying out the disclosure understood by those skilled in the relevant fields are intended to be within the scope of the following claims. All publications and patents mentioned in the present application are herein incorporated by reference.

5

CLAIMS

We Claim:

1. A method of sequencing a nucleic acid library where the concentration of the library is unknown, comprising:

a) contacting a nucleic acid library that has not been quantitated with a plurality of sequencing reaction components, wherein at least one of said reaction components is present at a limiting concentration; and

b) performing said sequencing reaction.

2. The method of claim 1, wherein said reaction components are selected from polymerase, dNTPs, and sequencing primers.

3. The method of claim 1, wherein said sequencing is next generation sequencing.

4. The method of claim 3, wherein said next generation sequencing is single molecule sequencing.

5. The method of claim 4, wherein said sequencing reaction is performed on a solid support comprising a plurality of reaction sites.

6. The method of claim 5, wherein at least one of said reaction components is present at a concentration of one molecule of reaction component per said reaction site.

7. The method of claim 1, wherein said nucleic acid library is prepared by whole genome amplification.

8. The method of claim 7, wherein said whole genome amplification is performed on genomic DNA with at least one amplification reagent present in limiting quantities, and wherein the concentration of said genomic DNA is unknown.

9. The method of claim 1, wherein said nucleic acid library has at least 100,000 target sequences.

10. The method of claim 1, wherein said nucleic acid library has at least 1,000,000 target sequences

11. The method of claim 1, wherein said nucleic acid library is suspected of having at least 100,000 target sequences.

12. The method of claim 1, wherein said nucleic acid library is suspected of having at least 1,000,000 target sequences.

13. A method of sequencing a nucleic acid library, comprising:

a) contacting a nucleic acid library with a plurality of sequencing reaction components, wherein said at least one of said reaction components is present at a molar concentration less than the number of nucleic acid targets in said nucleic acid library; and

b) performing said sequencing reaction.

14. The method of claim 13, wherein where the concentration of nucleic acids in said nucleic acid library is not quantitated.

15. The method of claim 13, wherein said reaction components are selected from polymerase, dNTPs, and sequencing primers.

16. The method of claim 13, wherein said sequencing is next generation sequencing.

17. The method of claim 16, wherein said next generation sequencing is single molecule sequencing.

18. The method of claim 17, wherein said sequencing reaction is performed on a solid support comprising a plurality of reaction sites.

19. The method of claim 18, wherein said reaction sites are wells.

20. The method of claim 19, wherein said wells are zero-mode waveguides.

21. A system comprising:

a) a container comprising a nucleic acid sequencing library that has not been quantitated and a plurality of reaction components, wherein at least one of said reaction components is present at limiting concentration; and

b) a nucleic acid sequencing device or system.

22. The system of claim 21, wherein said reaction components are selected from polymerase, dNTPs, and sequencing primers.

23. A system comprising:

a) a container comprising a first number of molecules of sequencing library nucleic acid targets and a second number of polymerase molecules, wherein said second number of molecules is less than said first number of molecules; and

b) a nucleic acid sequencing device or system.

24. The system of Claim 23, wherein sad second number of molecules is at least two times less than said first number of molecules.

25. The system of Claim 23, wherein sad second number of molecules is at least four times less than said first number of molecules.

26. The system of claim 21 or 22, wherein said sequencing device or system is a next generation sequencing devices or system.

27. The system of claim 26, wherein said sequencing device or system comprises a solid support comprising a plurality of reaction sites.

28. The system of claim 27, wherein said reaction sites are wells.

29. The system of claim 28, wherein said wells are zero-mode waveguides.

30. The system of claim 21, wherein at least one of said reaction components is present at a concentration of one molecule of reaction component per said reaction site.

31. The system of claim 21 or 22, wherein said nucleic acid library has or is suspected of having at least 100,000 target sequences.

32. The system of claim 21 or 22, wherein said nucleic acid library has or is suspected of having at least 1,000,000 target sequences.

FIG. 1

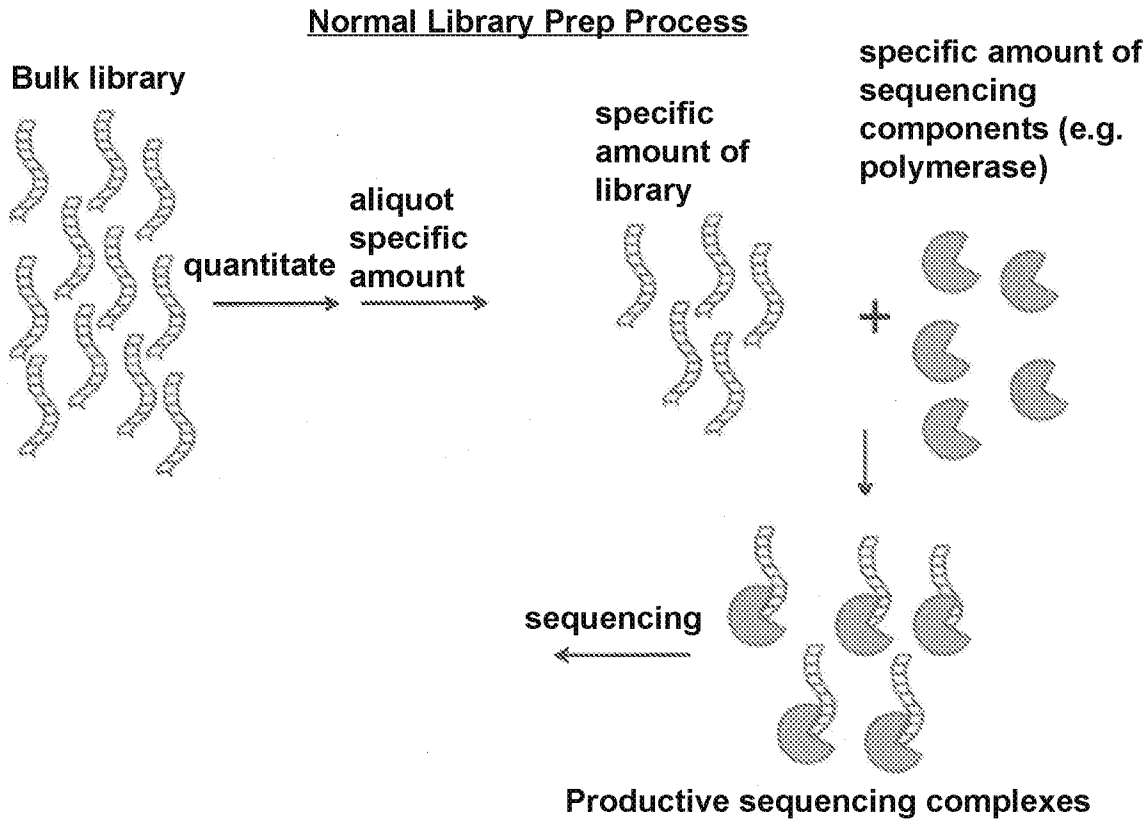

Normal Library Prep Process
Bulk library
quantitate
aliquot specific amount
specific amount of library
specific amount of sequencing components (e.g. polymerase)
sequencing
Productive sequencing complexes

FIG. 2

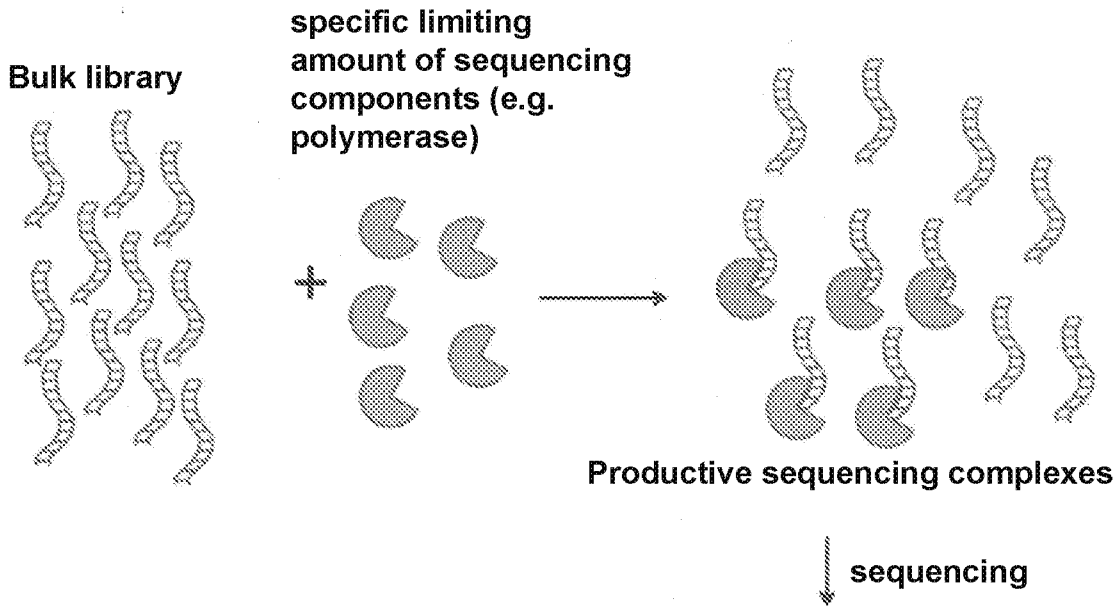
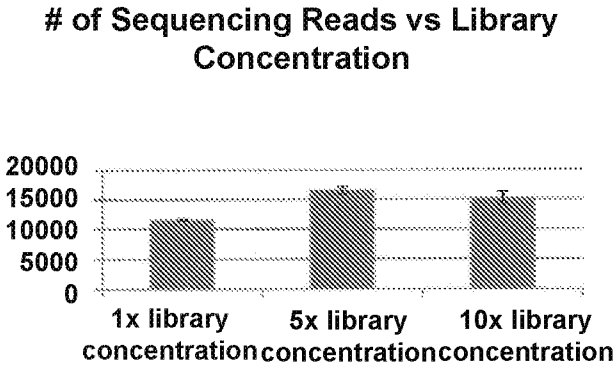
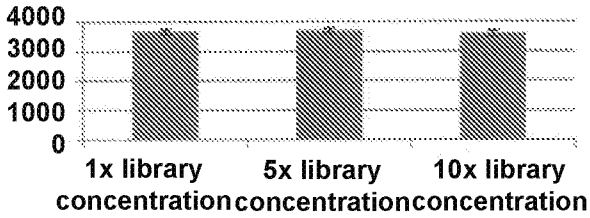

Bulk library
specific limiting amount of sequencing components (e.g. polymerase)
Productive sequencing complexes
sequencing

FIG. 3

A.


# of Sequencing Reads vs Library Concentration
20000
15000
10000
5000
0
1x library concentration
5x library concentration
10x library concentration

B.


Average Readlength vs Library Concentration
4000
3000
2000
1000
0
1x library concentration
5x library concentration
10x library concentration

C.

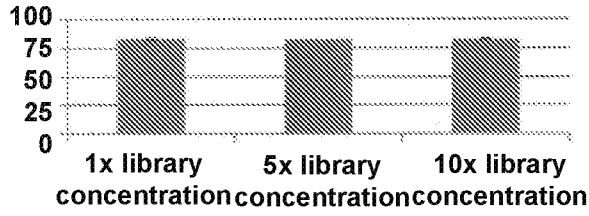
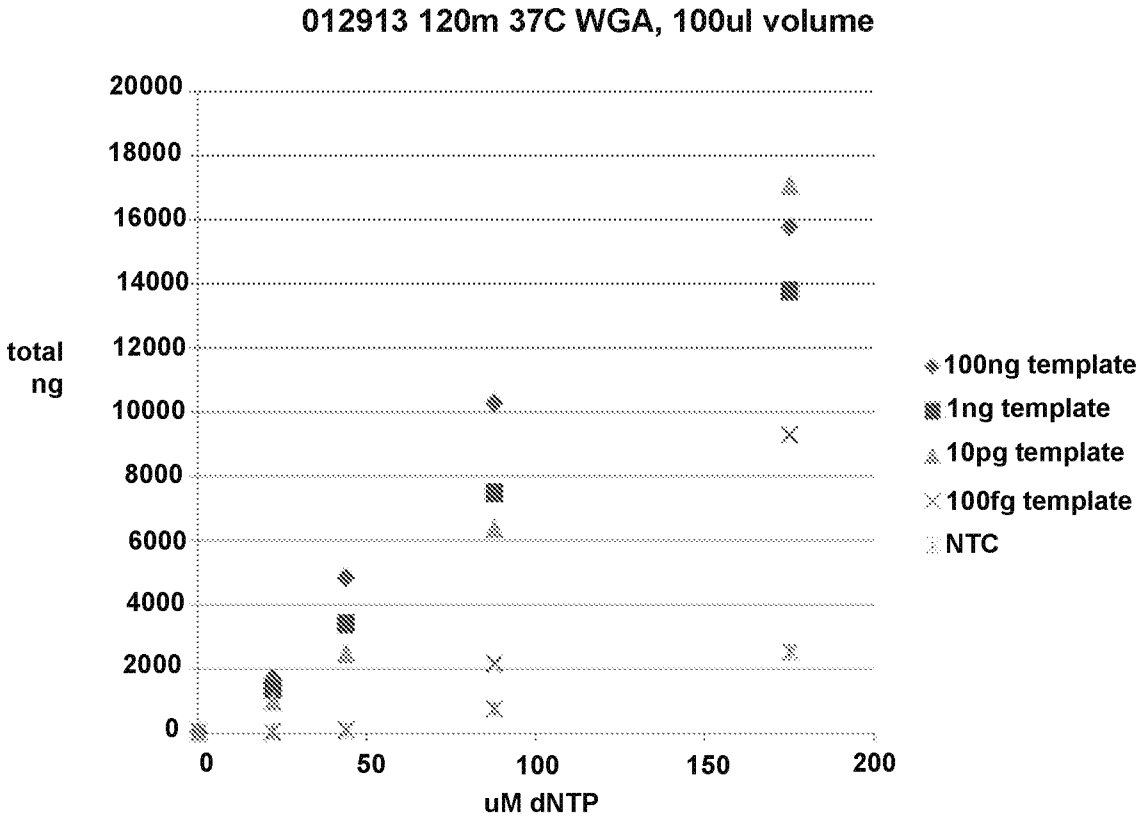

Read Quality vs Library Concentration
100
75
50
25
0
1x library concentration
5x library concentration
10x library concentration

FIG. 4


012913 120m 37C WGA, 100ul volume
total ng
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
50
100
150
200
uM dNTP
100ng template
1ng template
10pg template
100fg template
NTC

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/60414

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68, C12P 19/34, C07H 21/02 (2016.01)
CPC - C12Q 1/6883, C12Q 1/686, A61K 38/00
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)
IPC(8)- C12Q 1/68, C12P 19/34, C07H 21/02 (2016.01)
CPC- C12Q 1/6883, C12Q 1/686, A61K 38/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 435/6.1, 435/91.2, 536/23.1 (keyword search, terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (USPT, PGPB, EPAB, JPAB), Google Patents/Scholar
Search Terms Used: Nucleic library, polymerase, dNTPs, primer, limiting, reduced, exhaust, one-sided PCR, asymmetric amplification, whole genome amplification, next generation sequencing, single molecule sequencing

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	"Sequencing of Genomic DNA by Combined Amplification and Cycle Sequencing Reaction" by Murphy et al. (hereinafter 'Murphy') Clinical Chemistry 51(1) 35-39 (2005) abstract, pg 36, col 1, para 1, pg 37, col 1, para 1, pg 38, col 2, para 2	1-20
Y	US 2010/0021899 A1 (Ikebukuro et al.) 28 January 2010 (28.01.2010) para [0026], [0029], [0079]	1-20
Y	US 2014/0106360 A1 (ABBOT MOLECULAR INC.) 17 April 2014 (17.04.2014) para [0009], [0010], [0012]	3-8, 16-20
Y	US 2014/0017680 A1 (PACIFIC BIOSCIENCE OF CALIFORNIA, INC.) 16 January 2014 (16.01.2014) abstract, para [0060]	20

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"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

Date of the actual completion of the international search	Date of mailing of the international search report
16 March 2016	29 MAR 2016

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/60414

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-20, drawn to a method of sequencing a nucleic acid library.

Group II: Claims 21-32, drawn to a system involving DNA sequencing device.

- Please see extra sheet for continuation -

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-20

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/60414

Continuation of: Box NO III. Observations where unity of invention is lacking

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I has the special technical feature of a method of sequencing a nucleic acid library that has not been quantitated, involving the steps of mixing the nucleic acid library with sequencing reagents, where at least one of the reagents (e.g., dNTP, primer, polymerase) is limiting, and performing a sequencing reaction, not required by Group II.

Group II has the special technical feature of a system that utilizes a nucleic acid sequencing device, not required by Group I.

Common Technical Feature:

The common technical feature of Groups I and II is a rate-limiting amount of at least one reagent in a sequencing reaction.

However, said common technical feature does not represent a contribution over the prior art, and is anticipated by the publication titled "Nucleic Acid Laboratory Automated DNA Sequencing Manual" by PNACL DNA Sequencing Lab (hereinafter "PNACL") [published January 2007 and available online at <URL: http://devbio.wustl.edu/pnacl/forms/Manual.03.19.12.pdf>]

Concerning the common technical feature, PNACL teaches "PDF pg 7 para 2; "if you are sure you have a single extension product based on gel analysis, there are two options for direct sequencing [?] You can optimize PCR conditions to limit the amounts of primers and dNTPs in the reaction so that most of the primers and dNTPs are exhausted during amplification of the fragment"; PNACL further teaches (PDF pg 15 para 2; " When sequencing new templates, always start by using the standard reaction protocol. If your signal levels are good, you can decrease the amount of BigDye premix you use in subsequent reactions. [Big Dye premix dilution series for optimizing are indicated]

As the common technical feature was known in the art at the time of the invention, this cannot be considered a common special technical feature that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I and II lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.