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(54) **Titre : COMPOSITIONS ET PROCEDES DE SEQUENCAGE PAR PAIRES**
(54) **Title: COMPOSITIONS AND METHODS FOR PAIRWISE SEQUENCING**

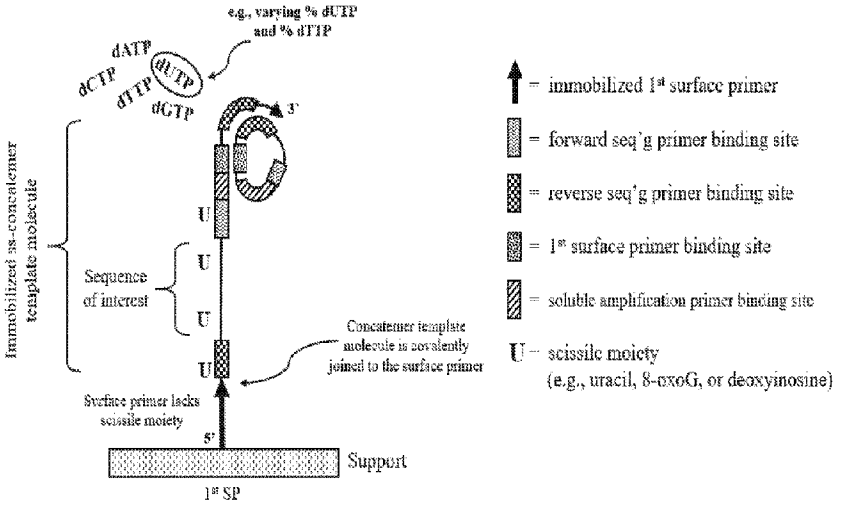

FIG. 26

(57) **Abrégé/Abstract:**
The present disclosure provides compositions and methods that employ the compositions for conducting pairwise sequencing and for generating concatemer template molecules for pairwise sequencing. The concatemers can be generated using a rolling circle amplification reaction which is conducted either on-support, or conducted in-solution and then distributed onto a support. The rolling circle amplification reaction generates concatemers containing tandem copies of a sequence of interest and at least one universal adaptor sequence. An increase in the number of tandem copies in a given concatemer increases the number of sites along the concatemer for hybridizing to multiple sequencing primers which serve as multiple initiation sites for polymerase-catalyzed sequencing reactions. When the sequencing reaction employs detectably labeled nucleotides and/or detectably labeled multivalent molecules (e.g., having nucleotide units), the signals emitted by the nucleotides or nucleotide units that participate in the parallel sequencing reactions along the concatemer yields an increased signal intensity for each concatemer.

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(54) Title: COMPOSITIONS AND METHODS FOR PAIRWISE SEQUENCING

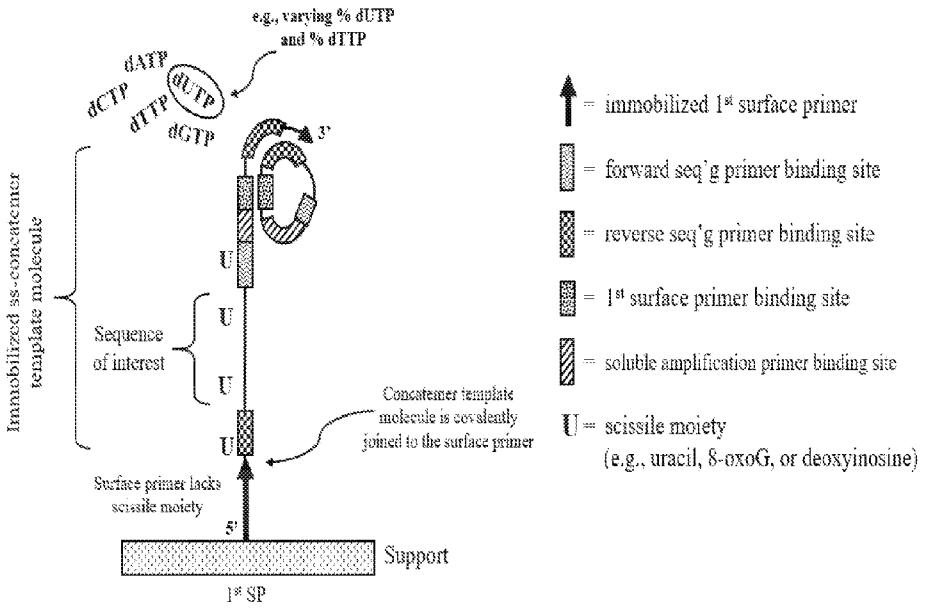

FIG. 26

(57) Abstract: The present disclosure provides compositions and methods that employ the compositions for conducting pairwise sequencing and for generating concatemer template molecules for pairwise sequencing. The concatemers can be generated using a rolling circle amplification reaction which is conducted either on-support, or conducted in-solution and then distributed onto a support. The rolling circle amplification reaction generates concatemers containing tandem copies of a sequence of interest and at least one universal adaptor sequence. An increase in the number of tandem copies in a given concatemer increases the number of sites along the concatemer for hybridizing to multiple sequencing primers which serve as multiple initiation sites for polymerase-catalyzed sequencing reactions. When the sequencing reaction employs detectably labeled nucleotides and/or detectably labeled multivalent molecules (e.g., having nucleotide units), the signals emitted by the nucleotides or nucleotide units that participate in the parallel sequencing reactions along the concatemer yields an increased signal intensity for each concatemer.

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CONTENANT LES PAGES 1 À 166

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THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

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COMPOSITIONS AND METHODS FOR PAIRWISE SEQUENCING

[0001] Throughout this application various publications, patents, and/or patent applications are referenced. The disclosures of the publications, patents and/or patent applications are hereby incorporated by reference in their entireties into this application in order to more fully describe the state of the art to which this disclosure pertains.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims the benefit of U.S. Provisional Patent Application No. 63/212,059, filed June 17, 2021; U.S. Patent Application No. 17/377,284, filed July 15, 2021, now issued as U.S. Patent No. 11,220,707; U.S. Patent Application No. 17/377,285, filed July 15, 2021, now issued as U.S. Patent No. 11,236,388; U.S. Patent Application No. 17/377,279, filed July 15, 2021; U.S. Patent Application No. 17/377,283, filed July 15, 2021; U.S. Patent Application No. 17/521,239, filed November 8, 2021; U.S. Patent Application No. 17/554,396, filed December 17, 2021, the contents of each of which are incorporated by reference herein in their entireties.

TECHNICAL FIELD

[0003] The present disclosure provides compositions and methods that employ the compositions for conducting pairwise sequencing and for generating concatemer template molecules for pairwise sequencing.

BACKGROUND OF THE INVENTION

[0004] Polynucleotide sequencing technology has applications in biomedical research and healthcare settings. Improved methods of polynucleotide require enhanced surface chemistry, on-support polynucleotide amplification, and base calling. Currently, these elements produce barriers in existing sequencing technology that result in limits in throughput and poor signal-to-noise ratio, and ultimately to increased costs associated with polynucleotide sequencing.

[0005] There exists a need for new polynucleotide sequencing methods with improved surface chemistry, on-support amplification, and base calling. The present disclosure provides methods and compositions to improve sequencing of polynucleotides.

SUMMARY OF THE INVENTION

[0006] The present disclosure provides a method for pairwise sequencing, comprising: a) providing a plurality of immobilized single stranded nucleic acid concatemer template molecules each comprising at least one nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein individual concatemer template molecules in the plurality are immobilized to a first surface primer that is immobilized to a support, and wherein the immobilized first surface primer lacks a nucleotide having a scissile moiety; b) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon; c) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction; d) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized surface primers; and e) sequencing the plurality of retained forward extension strands thereby generating a plurality of extended reverse sequencing primer strands, wherein individual retained forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

[0007] In some embodiments, the individual concatemer template molecules in the plurality are covalently joined to an immobilized first surface primer. In some embodiments, the individual concatemer template molecules in the plurality are hybridized to an immobilized first surface primer. In some embodiments, the individual immobilized concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of (i) two or more copies of a universal binding sequence for a

soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, (viii) two or more copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[0008] In some embodiments, the sequencing of step (b) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions. In some embodiments, the sequencing of step (e) comprises hybridizing a plurality of soluble reverse sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

[0009] In some embodiments, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some embodiments, at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer. In some embodiments, the plurality of immobilized second surface primers have 3' OH extendible ends. In some embodiments, the plurality of immobilized second surface primers have 3' non-extendible ends. In some embodiments, the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

[0010] The present disclosure also provides a method for pairwise sequencing, comprising: a) providing a support having a plurality of a first surface primer immobilized thereon wherein each of the first surface primers have a 3' extendible end and lack a nucleotide having a scissile moiety; b) generating a plurality of immobilized single stranded nucleic acid concatemer template molecules by hybridizing a plurality of single-stranded circular nucleic acid library molecules to the plurality of immobilized first surface primers and conducting a rolling circle amplification reaction with a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety that can be cleaved to generate an abasic site, thereby generating a plurality of immobilized

single stranded nucleic acid concatemer template molecules having at least one nucleotide with a scissile moiety, wherein individual single stranded nucleic acid concatemer template molecules are covalently joined to an immobilized first surface primer, c) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon; d) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction; e) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers; and f) sequencing the plurality of retained forward extension strands thereby generating a plurality of extended reverse sequencing primer strands, wherein individual forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

[0011] In some embodiments, each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest and wherein the individual library molecules further comprise any one or any combination of two or more of (i) a universal binding sequence for a soluble forward sequencing primer, (ii) a universal binding sequence for a soluble reverse sequencing primer, (iii) a universal binding sequence for an immobilized first surface primer, (iv) a universal binding sequence for an immobilized second surface primer, (v) a universal binding sequence for a first soluble amplification primer, (vi) a universal binding sequence for a second soluble amplification primer, (vii) a universal binding sequence for a soluble compaction oligonucleotide, (viii) a sample barcode sequence and/or (ix) a unique molecular index sequence.

[0012] In some embodiments, the individual immobilized single stranded nucleic acid concatemer template molecules generated by the rolling circle amplification reaction comprise two or more copies of a sequence of interest and wherein the individual immobilized concatemer

template molecules further comprise any one or any combination of two or more of (i) two or more copies of a universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, (viii) two or more copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[0013] In some embodiments, the sequencing of step (c) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions. In some embodiments, the sequencing of step (f) comprises hybridizing a plurality of soluble reverse sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

[0014] In some embodiments, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some embodiments, at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer. In some embodiments, the plurality of immobilized second surface primers have 3' OH extendible ends. In some embodiments, the plurality of immobilized second surface primers have 3' non-extendible ends. In some embodiments, the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

[0015] The present disclosure also provides a method for pairwise sequencing, comprising: a) contacting in-solution a plurality of single-stranded circular nucleic acid library molecules to a plurality of first soluble amplification primers, a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety that can be cleaved to generate an abasic site, under a condition suitable to form a plurality of library-primer duplexes and suitable for conducting a rolling circle amplification reaction, thereby generating a plurality of single stranded nucleic acid concatemers having at

least one nucleotide with a scissile moiety; b) distributing the rolling circle amplification reaction onto a support having a plurality of the first surface primers immobilized thereon, under a condition suitable for hybridizing one or more portions of individual single stranded concatemers to one or more immobilized first surface primers, wherein each of the first surface primers lack a nucleotide having a scissile moiety; c) continuing the rolling circle amplification reaction on the support to generate a plurality of immobilized concatemer template molecules; d) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon; e) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction; f) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers; and g) sequencing the plurality of retained forward extension strands thereby generating a plurality of extended reverse sequencing primer strands wherein individual forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

[0016] In some embodiments, each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest and wherein the individual library molecules further comprise any one or any combination of two or more of (i) a universal binding sequence for a soluble forward sequencing primer, (ii) a universal binding sequence for a soluble reverse sequencing primer, (iii) a universal binding sequence for an immobilized first surface primer, (iv) a universal binding sequence for an immobilized second surface primer, (v) a universal binding sequence for a first soluble amplification primer, (vi) a universal binding sequence for a second soluble amplification primer, (vii) a universal binding sequence for a soluble compaction oligonucleotide, (viii) a sample barcode sequence and/or (ix) a unique molecular index sequence.

[0017] In some embodiments, individual immobilized single stranded nucleic acid concatemer template molecules generated by the rolling circle amplification reaction comprise two or more copies of a sequence of interest and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of (i) two or more copies of a universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, (viii) two or more copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[0018] In some embodiments, the sequencing of step (d) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions. In some embodiments, the sequencing of step (g) comprises hybridizing a plurality of soluble reverse sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

[0019] In some embodiments, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some embodiments, at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer. In some embodiments, the plurality of immobilized second surface primers have 3' OH extendible ends. In some embodiments, the plurality of immobilized second surface primers have 3' non-extendible ends. In some embodiments, the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

[0020] The present disclosure provides a method for pairwise sequencing, comprising: a) providing a support having a plurality of a first surface primer immobilized thereon wherein individual first surface primers in the plurality comprise a first portion (SP1-A) and a second portion (SP1-B), and the individual first surface primers comprising a 3' extendible end and

lacking a nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the first surface primer; b) contacting the plurality of the first surface primers with a plurality of single stranded linear nucleic acid library molecules, each library molecule having at the 5' end a universal sequence (SP1-A') that binds the first portion of the immobilized first surface primer, and the library molecules each having at the 3' end a universal sequence (SP1-B') that binds the second portion of the immobilized first surface primer, wherein the contacting is conducted under a condition suitable for hybridizing individual library molecules to an immobilized first surface primer to form a circularized library molecule having a gap or nick between the 5' and 3' ends of the circularized library molecule; c) enzymatically closing the gap or nick thereby forming individual covalently closed circular molecules that are hybridized to an immobilized first surface primer; d) generating a plurality of immobilized single stranded nucleic acid concatemer template molecules by conducting a rolling circle amplification reaction with a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety that can be cleaved to generate an abasic site, thereby generating a plurality of immobilized single stranded nucleic acid concatemer template molecules having at least one nucleotide with a scissile moiety, wherein individual single stranded nucleic acid concatemer template molecules are covalently joined to an immobilized first surface primer; e) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon; f) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction; g) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers; and h) sequencing the plurality of retained forward extension strands thereby generating a plurality of extended reverse sequencing primer strands, wherein

individual forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

[0021] In some embodiments, individual linear library molecules in the plurality comprise a sequence of interest and the library molecules further comprise any one or any combination of two or more of: (i) a universal binding sequence for a soluble forward sequencing primer, (ii) a universal binding sequence for a soluble reverse sequencing primer, (iii) a universal binding sequence for a first portion of an immobilized first surface primer (SP1-A), (iv) a universal binding sequence for a second portion of an immobilized first surface primer (SP1-B), (v) a universal binding sequence for an immobilized second surface primer, (vi) a universal binding sequence for a first soluble amplification primer, (vii) a universal binding sequence for a second soluble amplification primer, (viii) a universal binding sequence for a soluble compaction oligonucleotide, (ix) a sample barcode sequence and/or (x) a unique molecular index sequence.

[0022] In some embodiments, individual immobilized single stranded nucleic acid concatemer template molecules generated by the rolling circle amplification reaction comprise two or more copies of a sequence of interest and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of (i) two or more copies of a universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for a first portion of an immobilized first surface primer (SP1-A), (iv) two or more copies of a universal binding sequence for a second portion of an immobilized first surface primer (SP1-B), (v) two or more copies of a universal binding sequence for an immobilized second surface primer, (vi) two or more copies of a universal binding sequence for a first soluble amplification primer, (vii) two or more copies of a universal binding sequence for a second soluble amplification primer, (viii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, (ix) two or more copies of a sample barcode sequence and/or (x) two or more copies of a unique molecular index sequence.

[0023] In some embodiments, the sequencing of step (e) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions. In some embodiments, the sequencing of step (h) comprises hybridizing a plurality of soluble reverse sequencing primers to

the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

[0024] In some embodiments, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some embodiments, at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer. In some embodiments, the plurality of immobilized second surface primers have 3' OH extendible ends. In some embodiments, the plurality of immobilized second surface primers have 3' non-extendible ends. In some embodiments, the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

[0025] In some embodiments, the closing the gap in the circularized library molecule comprises conducting a polymerase-catalyzed gap fill-in reaction using the immobilized first surface primer as a template molecule, and ligating the nick to form a covalently closed circular molecule, wherein individual covalently closed circular molecules are hybridized to an immobilized first surface primer. In some embodiments, the closing the nick in the circularized library molecule comprises conducting a ligation reaction to form a covalently closed circular molecule, and wherein individual covalently closed circular molecules are hybridized to an immobilized first surface primer.

[0026] The present disclosure provides a method for pairwise sequencing, comprising: a) providing a plurality of immobilized single stranded nucleic acid concatemer template molecules each lacking a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein individual concatemer template molecules in the plurality are immobilized to a first surface primer that is immobilized to a support, and wherein the immobilized first surface primer lacks a nucleotide having a scissile moiety; b) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon; c) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands by conducting a primer extension reaction with a plurality of soluble amplification primers and a plurality of strand-displacing polymerases to generate a plurality of forward

extension strands and a plurality of partially displaced forward extension strands that are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized amplicons, and the primer extension reaction generates a plurality of detached forward extension strands (e.g., that are not hybridized to the immobilized concatemer template molecules); and d) sequencing the plurality of immobilized partially displaced forward extension strands thereby generating a first plurality of extended reverse sequencing primer strands, and sequencing the plurality of immobilized detached forward extension strands thereby generating a second plurality of extended reverse sequencing primer strands, wherein individual immobilized partially displaced forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon, and wherein in individual immobilized detached forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

[0027] In some embodiments, individual concatemer template molecules in the plurality are covalently joined to an immobilized first surface primer. In some embodiments, individual concatemer template molecules in the plurality are hybridized to an immobilized first surface primer. In some embodiments, individual immobilized concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of (i) two or more copies of a universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, (viii) two or more copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[0028] In some embodiments, the sequencing of step (b) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions. In some embodiments, the sequencing of step (d) comprises hybridizing a plurality of soluble reverse sequencing primers to

the plurality of immobilized partially displaced forward extension strands and the plurality of immobilized detached extended forward sequencing primer strands, and conducting one or more sequencing reactions.

[0029] In some embodiments, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some embodiments, at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer. In some embodiments, the plurality of immobilized second surface primers have 3' OH extendible ends. In some embodiments, the plurality of immobilized second surface primers have 3' non-extendible ends. In some embodiments, the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

[0030] The present disclosure also provides a method for pairwise sequencing, comprising: a) providing a support having a plurality of a first surface primer immobilized thereon wherein each of the first surface primers have a 3' extendible end and lack a nucleotide having a scissile moiety; b) generating a plurality of immobilized single stranded nucleic acid concatemer template molecules by hybridizing a plurality of single-stranded circular nucleic acid library molecules to the plurality of immobilized first surface primers and conducting a rolling circle amplification reaction with a plurality of a strand displacing polymerase, and a plurality of nucleotides which lack a nucleotide having a scissile moiety that can be cleaved to generate an abasic site, thereby generating a plurality of immobilized single stranded nucleic acid concatemer template molecules, wherein individual single stranded nucleic acid concatemer template molecules are covalently joined to an immobilized first surface primer; c) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon; d) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands by conducting a primer extension reaction with a plurality of soluble amplification primers and a plurality of strand-displacing polymerases to generate a plurality of forward extension strands and a plurality of partially displaced forward extension strands that are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized

amplicons, and the primer extension reaction generates a plurality of detached forward extension strands (e.g., that are not hybridized to the immobilized concatemer template molecules); and e) sequencing the plurality of immobilized partially displaced forward extension strands thereby generating a first plurality of extended reverse sequencing primer strands, and sequencing the plurality of immobilized detached forward extension strands thereby generating a second plurality of extended reverse sequencing primer strands, wherein individual immobilized partially displaced forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon, and wherein in individual immobilized detached forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

[0031] In some embodiments, each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest, and wherein the individual library molecules further comprise any one or any combination of two or more of (i) a universal binding sequence for a soluble forward sequencing primer, (ii) a universal binding sequence for a soluble reverse sequencing primer, (iii) a universal binding sequence for an immobilized first surface primer, (iv) a universal binding sequence for an immobilized second surface primer, (v) a universal binding sequence for a first soluble amplification primer, (vi) a universal binding sequence for a second soluble amplification primer, (vii) a universal binding sequence for a soluble compaction oligonucleotide, (viii) a sample barcode sequence and/or (ix) a unique molecular index sequence.

[0032] In some embodiments, individual immobilized single stranded nucleic acid concatemer template molecules generated by the rolling circle amplification reaction comprise two or more copies of a sequence of interest, wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of (i) two or more copies of a universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide,

(viii) two or more copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[0033] In some embodiments, the sequencing of step (c) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions. In some embodiments, the sequencing of step (e) comprises hybridizing a plurality of soluble reverse sequencing primers to the plurality of immobilized partially displaced forward extension strands and the plurality of immobilized detached extended forward sequencing primer strands, and conducting one or more sequencing reactions.

[0034] In some embodiments, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some embodiments, the at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer. In some embodiments, the plurality of immobilized second surface primers have 3' OH extendible ends. In some embodiments, the plurality of immobilized second surface primers have 3' non-extendible ends. In some embodiments, the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

[0035] The present disclosure also provides a method for pairwise sequencing, comprising: a) contacting in-solution a plurality of single-stranded circular nucleic acid library molecules to a plurality of first soluble amplification primers, a plurality of a strand displacing polymerase, and a plurality of nucleotides which lacks a nucleotide having a scissile moiety that can be cleaved to generate an abasic site, under a condition suitable to form a plurality of library-primer duplexes and suitable for conducting a rolling circle amplification reaction, thereby generating a plurality of single stranded nucleic acid concatemers; b) distributing the rolling circle amplification reaction onto a support having a plurality of the first surface primers immobilized thereon, under a condition suitable for hybridizing one or more portions of individual single stranded concatemers to one or more immobilized first surface primers, wherein each of the first surface primers lack a nucleotide having a scissile moiety; c) continuing the rolling circle amplification reaction on the support to generate a plurality of immobilized concatemer template molecules; d) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands wherein individual immobilized

concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon; e) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands by conducting a primer extension reaction with a plurality of a second soluble amplification primer and a plurality of strand-displacing polymerases to generate a plurality of forward extension strands and a plurality of partially displaced forward extension strands that are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized amplicons, and the primer extension reaction generates a plurality of detached forward extension strands (e.g., that are not hybridized to the immobilized concatemer template molecules); and f) sequencing the plurality of immobilized partially displaced forward extension strands thereby generating a first plurality of extended reverse sequencing primer strands, and sequencing the plurality of immobilized detached forward extension strands thereby generating a second plurality of extended reverse sequencing primer strands, wherein individual immobilized partially displaced forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon, and wherein in individual immobilized detached forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

[0036] In some embodiments, each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest, and wherein the individual library molecules further comprise any one or any combination of two or more of (i) a universal binding sequence for a soluble forward sequencing primer, (ii) a universal binding sequence for a soluble reverse sequencing primer, (iii) a universal binding sequence for an immobilized first surface primer, (iv) a universal binding sequence for an immobilized second surface primer, (v) a universal binding sequence for a first soluble amplification primer, (vi) a universal binding sequence for a second soluble amplification primer, (vii) a universal binding sequence for a soluble compaction oligonucleotide, (viii) a sample barcode sequence and/or (ix) a unique molecular index sequence.

[0037] In some embodiments, individual immobilized single stranded nucleic acid concatemer template molecules generated by the rolling circle amplification reaction comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of

(i) two or more copies of a universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, (viii) two or more copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[0038] In some embodiments, the sequencing of step (d) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions. In some embodiments, the sequencing of step (f) comprises hybridizing a plurality of soluble reverse sequencing primers to the plurality of immobilized partially displaced forward extension strands and the plurality of immobilized detached extended forward sequencing primer strands, and conducting one or more sequencing reactions.

[0039] In some embodiments, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some embodiments, at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer. In some embodiments, the plurality of immobilized second surface primers have 3' OH extendible ends. In some embodiments, the plurality of immobilized second surface primers have 3' non-extendible ends. In some embodiments, the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

[0040] The present disclosure also provides a method for pairwise sequencing, comprising: a) providing a plurality of immobilized single stranded nucleic acid concatemer template molecules each comprising at least one nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein individual concatemer template molecules in the plurality are immobilized to a first surface primer that is immobilized to a support, wherein the immobilized first surface primers include a nucleotide having a scissile moiety, wherein the support further comprises a plurality of immobilized second surface primers

which lack a nucleotide having a scissile moiety and have an extendible terminal 3'OH group, and wherein the immobilized concatemer template molecule comprises two or more copies of a universal binding sequence for an immobilized second surface primer (wherein the support comprises an excess of immobilized first and second surface primers compared to the number of immobilized concatemer template molecules); b) sequencing the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon; c) removing the extended forward sequencing primer strands and retaining the immobilized concatemer template molecules; d) generating a first plurality of immobilized forward extension strands by hybridizing at least one portion of individual immobilized concatemer template molecules to a second surface primer and conducting a primer extension reaction from the second surface primers that are hybridized to a portion of the immobilized concatemer template molecule to generate a plurality of forward extension strands having a sequence that is complementary to at least a portion of the immobilized concatemer template molecules and are covalently joined to an immobilized second surface primer; e) contacting the plurality of immobilized concatemer template molecules and the plurality of immobilized forward extension strands with a relaxing solution which comprises at least one chaotropic agent; f) dissociating the at least one portion of the immobilized concatemer template molecules from the immobilized second surface primers and retaining the immobilized forward extension strands, and re-hybridizing at least one portion of the immobilized concatemer template molecules to one of the immobilized second surface primers that are not covalently joined to a forward extension strand, wherein the dissociating and re-associating comprises a temperature ramp-up, a temperature plateau, and temperature ramp-down, and washing the relaxing solution from the support; g) contacting the re-hybridized immobilized concatemer template molecules with an amplification solution and conducting a primer extension reaction from the second surface primers that are re-hybridized to a portion of the immobilized concatemer template molecules to generate a plurality of newly synthesized forward extension strands having a sequence that is complementary to at least a portion of the immobilized concatemer template molecules and are covalently joined to an immobilized second surface primer; h) repeating steps (e) – (g) at least once; i) removing the retained immobilized

concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules and the immobilized first surface primers at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites thereby generating a plurality of gap-containing nucleic acid molecules while retaining the plurality of immobilized forward extension strands and retaining the plurality of immobilized second surface primers; and j) sequencing the plurality of retained immobilized forward extension strands with a plurality of soluble reverse sequencing primers thereby generating a plurality of extended reverse sequencing primer strands.

[0041] In some embodiments, individual concatemer template molecules in the plurality are covalently joined to an immobilized first surface primer. In some embodiments, individual concatemer template molecules in the plurality are hybridized to an immobilized first surface primer. In some embodiments, individual immobilized concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of (i) two or more copies of a universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, (viii) two or more copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[0042] The present disclosure also provides a method for pairwise sequencing, comprising: a) providing a support having a plurality of first and second surface primers immobilized thereon, wherein the first surface primers have a scissile moiety that can be cleaved to generate an abasic site, and wherein the second surface primers lack a nucleotide having a scissile moiety and the second surface primers have an extendible terminal 3'OH group; b) generating a plurality of immobilized single stranded nucleic acid concatemer template molecules by hybridizing a plurality of single-stranded circular nucleic acid library molecules to the plurality of immobilized first surface primers and conducting a rolling circle amplification reaction with a plurality of a

strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a plurality of nucleotides having a scissile moiety that can be cleaved to generate an abasic site, thereby generating a plurality of immobilized single stranded nucleic acid concatemer template molecules having at least one nucleotide with a scissile moiety, wherein individual single stranded nucleic acid concatemer template molecules are covalently joined to an immobilized first surface primer; c) sequencing the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon; d) removing the extended forward sequencing primer strands and retaining the immobilized concatemer template molecules; e) generating a first plurality of immobilized forward extension strands by hybridizing at least one portion of individual immobilized concatemer template molecules to a second surface primer and conducting a primer extension reaction from the second surface primers that are hybridized to a portion of the immobilized concatemer template molecule to generate a plurality of forward extension strands having a sequence that is complementary to at least a portion of the immobilized concatemer template molecules and are covalently joined to an immobilized second surface primer; f) contacting the plurality of immobilized concatemer template molecules and the plurality of immobilized forward extension strands with a relaxing solution which comprises at least one chaotropic agent; g) dissociating the at least one portion of the immobilized concatemer template molecules from the immobilized second surface primers and retaining the immobilized forward extension strands, and re-hybridizing at least one portion of the immobilized concatemer template molecules to one of the immobilized second surface primers that are not covalently joined to a forward extension strand, wherein the dissociating and re-associating comprises a temperature ramp-up, a temperature plateau, and temperature ramp-down, and washing the relaxing solution from the support; h) contacting the re-hybridized immobilized concatemer template molecules with an amplification solution and conducting a primer extension reaction from the second surface primers that are re-hybridized to a portion of the immobilized concatemer template molecules to generate a plurality of newly synthesized forward extension strands having a sequence that is complementary to at least a portion of the immobilized concatemer template molecules and are covalently joined to an immobilized second surface primer; i) repeating steps

(f) – (h) at least once; j) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules and the immobilized first surface primers at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing nucleic acid molecules while retaining the plurality of immobilized forward extension strands and retaining the plurality of immobilized second surface primers; and k) sequencing the plurality of retained immobilized forward extension strands with a plurality of soluble reverse sequencing primers thereby generating a plurality of extended reverse sequencing primer strands.

[0043] In some embodiments, each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest and wherein the individual library molecules further comprise any one or any combination of two or more of (i) a universal binding sequence for a soluble forward sequencing primer, (ii) a universal binding sequence for a soluble reverse sequencing primer, (iii) a universal binding sequence for an immobilized first surface primer, (iv) a universal binding sequence for an immobilized second surface primer, (v) a universal binding sequence for a first soluble amplification primer, (vi) a universal binding sequence for a second soluble amplification primer, (vii) a universal binding sequence for a soluble compaction oligonucleotide, (viii) a sample barcode sequence and/or (ix) a unique molecular index sequence.

[0044] In some embodiments, individual immobilized concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of (i) two or more copies of a universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, (viii) two or more copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[0045] The present disclosure also provides a method for pairwise sequencing, comprising: a) contacting in-solution a plurality of single-stranded circular nucleic acid library molecules to a plurality of first soluble amplification primers, a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a plurality of nucleotides having a scissile moiety that can be cleaved to generate an abasic site, under a condition suitable to form a plurality of library-primer duplexes and suitable for conducting a rolling circle amplification reaction, thereby generating a plurality of single stranded nucleic acid concatemers having at least one nucleotide with a scissile moiety; b) distributing the rolling circle amplification reaction onto a support having a plurality of the first surface primers immobilized thereon, under a condition suitable for hybridizing one or more portions of individual single stranded concatemers to one or more immobilized first surface primers, wherein the immobilized first surface primers include a nucleotide having a scissile moiety, wherein the support further comprises a plurality of immobilized second surface primers which lack a nucleotide having a scissile moiety and have an extendible terminal 3'OH group; c) continuing the rolling circle amplification reaction on the support in the presence of a plurality of nucleotides which include a plurality of nucleotides having a scissile moiety to generate a plurality of immobilized concatemer template molecules; d) sequencing the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon; e) removing the extended forward sequencing primer strands and retaining the immobilized concatemer template molecules; f) generating a first plurality of immobilized forward extension strands by hybridizing at least one portion of individual immobilized concatemer template molecules to a second surface primer and conducting a primer extension reaction from the second surface primers that are hybridized to a portion of the immobilized concatemer template molecule to generate a plurality of forward extension strands having a sequence that is complementary to at least a portion of the immobilized concatemer template molecules and are covalently joined to an immobilized second surface primer; g) contacting the plurality of immobilized concatemer template molecules and the plurality of immobilized forward extension strands with a relaxing solution which comprises at least one chaotropic agent; h) dissociating the at least one portion of the immobilized concatemer template molecules from

the immobilized second surface primers and retaining the immobilized forward extension strands, and re-hybridizing at least one portion of the immobilized concatemer template molecules to one of the immobilized second surface primers that are not covalently joined to a forward extension strand, wherein the dissociating and re-associating comprises a temperature ramp-up, a temperature plateau, and temperature ramp-down, and washing the relaxing solution from the support; i) contacting the re-hybridized immobilized concatemer template molecules with an amplification solution and conducting a primer extension reaction from the second surface primers that are re-hybridized to a portion of the immobilized concatemer template molecules to generate a plurality of newly synthesized forward extension strands having a sequence that is complementary to at least a portion of the immobilized concatemer template molecules and are covalently joined to an immobilized second surface primer; j) repeating steps (g) – (i) at least once; k) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules and the immobilized first surface primers at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing nucleic acid molecules while retaining the plurality of immobilized forward extension strands and retaining the plurality of immobilized second surface primers; and l) sequencing the plurality of retained immobilized forward extension strands with a plurality of soluble reverse sequencing primers thereby generating a plurality of extended reverse sequencing primer strands.

[0046] In some embodiments, each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest and wherein the individual library molecules further comprise any one or any combination of two or more of (i) a universal binding sequence for a soluble forward sequencing primer, (ii) a universal binding sequence for a soluble reverse sequencing primer, (iii) a universal binding sequence for an immobilized first surface primer, (iv) a universal binding sequence for an immobilized second surface primer, (v) a universal binding sequence for a first soluble amplification primer, (vi) a universal binding sequence for a second soluble amplification primer, (vii) a universal binding sequence for a soluble compaction oligonucleotide, (viii) a sample barcode sequence and/or (ix) a unique molecular index sequence.

[0047] In some embodiments, individual immobilized concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual

immobilized concatemer template molecules further comprise any one or any combination of two or more of (i) two or more copies of a universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, (viii) two or more copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[0048] In any of the foregoing or related embodiments, the support comprises a planar substrate which comprises glass, fused-silica, silicon, a polymer (e.g., polystyrene (PS), macroporous polystyrene (MPPS), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET)), or any combination thereof.

[0049] In any of the foregoing or related embodiments, the support comprises at least one hydrophilic polymer coating having a water contact angle of no more than 45 degrees, and wherein at least one of the hydrophilic polymer coatings comprising branched hydrophilic polymer having at least 4 branches.

[0050] In any of the foregoing or related embodiments, the 5' end of the plurality of first surface primers are immobilized to the support or immobilized to a coating on the support. In any of the foregoing or related embodiments, the plurality of first surface primers comprise modified oligonucleotide molecules having 2-10 phosphorothioate linkages at their 5' ends to confer resistance to nuclease degradation.

[0051] In any of the foregoing or related embodiments, the 5' end of the plurality of second surface primers are immobilized to the support or immobilized to a coating on the support. In some embodiments, the plurality of second surface primers comprise modified oligonucleotide molecules having 2-10 phosphorothioate linkages at their 5' ends to confer resistance to nuclease degradation.

[0052] In any of the foregoing or related embodiments, the immobilized concatemer template molecules comprise at least one nucleotide having a scissile moiety which comprises uridine, 8-oxo-7,8-dihydrogunine, or deoxyinosine.

[0053] In any of the foregoing or related embodiments, the nucleotides with a scissile moiety are located at randomly distributed positions in individual immobilized concatemer template molecules in the plurality.

[0054] In any of the foregoing or related embodiments, 0.01 – 30% of the thymidine nucleotides in the individual immobilized concatemer template molecules are replaced with uridine. In any of the foregoing or related embodiments, 0.01 – 30% of the guanosine nucleotides in the individual immobilized concatemer template molecules are replaced with 8-oxo-7,8-dihydrogunine or deoxyinosine.

[0055] In any of the foregoing or related embodiments, the soluble forward sequencing primer comprises a 3' OH extendible end and lacks a nucleotide having a scissile moiety. In any of the foregoing or related embodiments, the soluble reverse sequencing primer comprises a 3' OH extendible end and lacks a nucleotide having a scissile moiety.

[0056] In any of the foregoing or related embodiments, the first soluble amplification primer comprises a 3' OH extendible end and lacks a nucleotide having a scissile moiety. In any of the foregoing or related embodiments, the second soluble amplification primer comprises a 3' OH extendible end and lacks a nucleotide having a scissile moiety.

[0057] In any of the foregoing or related embodiments, the forward sequencing step comprises: a) contacting a plurality of sequencing polymerases to (i) a plurality of immobilized concatemer template molecules and (ii) a plurality of the soluble forward sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of complexed polymerases each comprising a sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises a immobilized concatemer template molecule hybridized to a soluble forward sequencing primer; b) contacting the plurality of complexed sequencing polymerases with a plurality of nucleotides under a condition suitable for binding at least one nucleotide to a complexed sequencing polymerase, wherein the plurality of nucleotides comprises at least one nucleotide analog labeled with a fluorophore and having a removable chain terminating moiety at the sugar 3' position; c) incorporating at least one nucleotide into the 3' end of the hybridized forward sequencing primers thereby generating a plurality of nascent

extended forward sequencing primers; and d) detecting the incorporated nucleotide and identifying the nucleo-base of the incorporated nucleotide.

[0058] In any of the foregoing or related embodiments, the reverse sequencing step comprises: a) contacting a plurality of sequencing polymerases to (i) a plurality of the retained forward extension strands and (ii) a plurality of the soluble reverse sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of complexed polymerases each comprising a sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises a retained forward extension strand hybridized to a soluble reverse sequencing primer; b) contacting the plurality of complexed sequencing polymerases with a plurality of nucleotides under a condition suitable for binding at least one nucleotide to a complexed sequencing polymerase, wherein the plurality of nucleotides comprises at least one nucleotide analog labeled with a fluorophore and having a removable chain terminating moiety at the sugar 3' position; c) incorporating at least one nucleotide into the 3' end of the hybridized reverse sequencing primers thereby generating a plurality of nascent extended reverse sequencing primers; and d) detecting the incorporated nucleotide and identifying the nucleo-base of the incorporated nucleotide.

[0059] In some embodiments, the reverse sequencing of step (a) comprises hybridizing the plurality of soluble reverse sequencing primers to the plurality of the retained forward extension strands in the presence of a high efficiency hybridization buffer which comprises: (i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer; (iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

[0060] In some embodiments, the reverse sequencing step comprises: a) contacting a plurality of sequencing polymerases to (i) a plurality of the immobilized partially displaced forward extension strands, (ii) a plurality of plurality of immobilized detached extended forward sequencing primer strands, and (iii) a plurality of the soluble reverse sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of complexed polymerases each comprising a sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises a soluble reverse sequencing primer hybridized to an

immobilized partially displaced forward extension strand or an immobilized detached extended forward sequencing primer strand; b) contacting the plurality of complexed sequencing polymerases with a plurality of nucleotides under a condition suitable for binding at least one nucleotide to a complexed sequencing polymerase, wherein the plurality of nucleotides comprises at least one nucleotide analog labeled with a fluorophore and having a removable chain terminating moiety at the sugar 3' position; c) incorporating at least one nucleotide into the 3' end of the hybridized reverse sequencing primers thereby generating a plurality of nascent extended reverse sequencing primers; and d) detecting the incorporated nucleotide and identifying the nucleo-base of the incorporated nucleotide.

[0061] In some embodiments, the reverse sequencing of step a) comprises hybridizing the plurality of soluble reverse sequencing primers to the plurality of the retained forward extension strands in the presence of a high efficiency hybridization buffer which comprises: (i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer; (iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

[0062] In any of the foregoing or related embodiments, the forward sequencing step and the reverse sequencing step comprises: 1) conducting a sequencing reaction at a position on the template molecule using multivalent molecules which bind but do not incorporate; 2) conducting a sequencing reaction at the same position on the template molecule using nucleotides with incorporation; and 3) repeating steps a) and b) at the next position on the template molecule.

[0063] In any of the foregoing or related embodiments, the forward sequencing step and the reverse sequencing step comprises: a) contacting a plurality of a first sequencing polymerase to (i) a plurality of nucleic acid template molecules and (ii) a plurality of soluble sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each comprising a first sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises the nucleic acid template molecule hybridized to the sequencing primer, wherein (1) the plurality of nucleic acid template molecules comprise a plurality of the immobilized concatemer template molecules and the plurality of soluble primers comprise a plurality of the soluble forward sequencing primers, or wherein (2)

the plurality of nucleic acid template molecules comprise a plurality of the retained forward extension strands and the plurality of soluble sequencing primers comprise a plurality of the soluble reverse sequencing primers; b) contacting the plurality of first complexed polymerases with a plurality of detectably labeled multivalent molecules to form a plurality of multivalent-complexed polymerases, under a condition suitable for binding complementary nucleotide units of the multivalent molecules to at least two of the plurality of first complexed polymerases thereby forming a plurality of multivalent-complexed polymerases, and the condition inhibits incorporation of the complementary nucleotide units into the sequencing primers of the plurality of multivalent-complexed polymerases, wherein individual multivalent molecules in the plurality of multivalent molecules comprise a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit; c) detecting the plurality of multivalent-complexed polymerases; and d) identifying the nucleo-base of the complementary nucleotide units that are bound to the plurality of first complexed polymerases in the plurality of multivalent-complexed polymerases, thereby determining the sequence of the nucleic acid template.

[0064] In some embodiments, the reverse sequencing of step comprises: hybridizing the plurality of soluble reverse sequencing primers to the plurality of the retained forward extension strands in the presence of a high efficiency hybridization buffer which comprises: (i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer; (iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

[0065] In some embodiments, the method further comprises: e) dissociating the plurality of multivalent-complexed polymerases and removing the plurality of first sequencing polymerases and their bound multivalent molecules, and retaining the plurality of nucleic acid duplexes; f) contacting the plurality of the retained nucleic acid duplexes of step (e) with a plurality of second sequencing polymerases, wherein the contacting is conducted under a condition suitable for binding the plurality of second sequencing polymerases to the plurality of the retained nucleic acid duplexes, thereby forming a plurality of second complexed polymerases each comprising a second sequencing polymerase bound to a retained nucleic acid duplex; g) contacting the

plurality of second complexed polymerases with a plurality of nucleotides, wherein the contacting is conducted under a condition suitable for binding complementary nucleotides from the plurality of nucleotides to at least two of the second complexed polymerases of step (f) thereby forming a plurality of nucleotide-complexed polymerases and the condition is suitable for promoting incorporation of the bound complementary nucleotides into the sequencing primers of the nucleotide-complexed polymerases; h) detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases; and d) identifying the nucleo-bases of the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

[0066] In some embodiments, the method further comprises: forming at least one avidity complex in step (b), the method comprising: a) binding a first sequencing primer, a first sequencing polymerase, and a first multivalent molecule to a first portion of a nucleic acid template molecule thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first sequencing polymerase; and b) binding a second sequencing primer, a second sequencing polymerase, and the first multivalent molecule to a second portion of the same nucleic acid template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the second multivalent molecule binds to the second sequencing polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms an avidity complex.

[0067] In some embodiments, (i) the first sequencing primer comprises a soluble forward sequencing primer and the nucleic acid template molecule comprises an immobilized concatemer template molecule, (ii) the second sequencing primer comprises a soluble forward sequencing primer and the nucleic acid template molecule comprises the same immobilized concatemer template molecule, and (iii) the first and second sequencing primers have the same sequence.

[0068] In some embodiments, wherein (i) the first sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises a retained forward extension strand, (ii) the second sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises the same retained forward extension strand, and (iii) the first and second sequencing primers have the same sequence.

[0069] In some embodiments, the method further comprises: forming at least one avidity complex in step (b), the method comprising: a) contacting a plurality of first sequencing

polymerases and a plurality of second sequencing primers with different portions of a nucleic acid template molecule to form at least first and second complexed polymerases on the same nucleic acid template molecule; b) contacting a plurality of multivalent molecules to the at least first and second complexed polymerases on the same nucleic acid template molecule, under conditions suitable to bind a single multivalent molecule from the plurality to the first and second complexed polymerases, wherein at least a first nucleotide unit of the single multivalent molecule is bound to the first complexed polymerase which includes a first sequencing primer hybridized to a first portion of the nucleic acid template molecule thereby forming a first binding complex, and wherein at least a second nucleotide unit of the single multivalent molecule is bound to the second complexed polymerase which includes a second sequencing primer hybridized to a second portion of the same nucleic acid template molecule thereby forming a second binding complex, wherein the contacting is conducted under a condition suitable to inhibit polymerase-catalyzed incorporation of the bound first and second nucleotide units in the first and second binding complexes, and wherein the first and second binding complexes which are bound to the same multivalent molecule forms an avidity complex; c) detecting the first and second binding complexes on the same nucleic acid template molecule, and d) identifying the first nucleotide unit in the first binding complex thereby determining the sequence of the first portion of the nucleic acid template molecule, and identifying the second nucleotide unit in the second binding complex thereby determining the sequence of the second portion of the same nucleic acid template molecule.

[0070] In some embodiments, (i) the plurality of first sequencing primers comprise a plurality of first soluble forward sequencing primers and the nucleic acid template molecule comprises an immobilized concatemer template molecule, (ii) the plurality of second sequencing primers comprise a plurality of second soluble forward sequencing primers and the nucleic acid template molecule comprises the same immobilized concatemer template molecule, and (iii) the plurality of first and second sequencing primers have the same sequence.

[0071] In some embodiments, (i) the plurality of first sequencing primers comprises a plurality of first soluble reverse sequencing primer and the nucleic acid template molecule comprises a retained forward extension strand, (ii) the plurality of second sequencing primers comprise a plurality of second soluble reverse sequencing primers and the nucleic acid template

molecule comprises the same retained forward extension strand, and (iii) the plurality of first and second sequencing primers have the same sequence.

[0072] In any of the foregoing or related embodiments, the forward sequencing step and the reverse sequencing step comprises: a) contacting a plurality of a first sequencing polymerase to (i) a plurality of nucleic acid template molecules and (ii) a plurality of soluble sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each comprising a first sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises the nucleic acid template molecule hybridized to the soluble sequencing primer, wherein (1) the plurality of nucleic acid template molecules comprise a plurality of the immobilized concatemer template molecules and the plurality of sequencing primers comprise a plurality of the soluble forward sequencing primers, or wherein (2) the plurality of nucleic acid template molecules comprise a plurality of immobilized partially displaced forward extension strands and the plurality of sequencing primers comprise a plurality of the soluble reverse sequencing primers, or wherein (3) the plurality of nucleic acid template molecules comprise a plurality of immobilized detached extended forward sequencing primer strands and the plurality of sequencing primers comprise a plurality of the soluble reverse sequencing primers; b) contacting the plurality of first complexed polymerases with a plurality of detectably labeled multivalent molecules to form a plurality of multivalent-complexed polymerases, under a condition suitable for binding complementary nucleotide units of the multivalent molecules to at least two of the plurality of first complexed polymerases thereby forming a plurality of multivalent-complexed polymerases, and the condition inhibits incorporation of the complementary nucleotide units into the sequencing primers of the plurality of multivalent-complexed polymerases, wherein individual multivalent molecules in the plurality of multivalent molecules comprise a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit; c) detecting the plurality of multivalent-complexed polymerases; and d) identifying the nucleo-base of the complementary nucleotide units that are bound to the plurality of first complexed polymerases in the plurality of multivalent-complexed polymerases, thereby determining the sequence of the nucleic acid template.

[0073] In any of the foregoing or related embodiments, the reverse sequencing step comprises: hybridizing the plurality of soluble reverse sequencing primers to the plurality of

immobilized partially displaced forward extension strands or the plurality of immobilized detached extended forward sequencing primer strands in the presence of a high efficiency hybridization buffer which comprises: (i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer; (iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

[0074] In some embodiments, the method further comprises: e) dissociating the plurality of multivalent-complexed polymerases and removing the plurality of first sequencing polymerases and their bound multivalent molecules, and retaining the plurality of nucleic acid duplexes; f) contacting the plurality of the retained nucleic acid duplexes of step (e) with a plurality of second sequencing polymerases, wherein the contacting is conducted under a condition suitable for binding the plurality of second sequencing polymerases to the plurality of the retained nucleic acid duplexes, thereby forming a plurality of second complexed polymerases each comprising a second sequencing polymerase bound to a retained nucleic acid duplex; g) contacting the plurality of second complexed polymerases with a plurality of nucleotides, wherein the contacting is conducted under a condition suitable for binding complementary nucleotides from the plurality of nucleotides to at least two of the second complexed polymerases of step (f) thereby forming a plurality of nucleotide-complexed polymerases and the condition is suitable for promoting incorporation of the bound complementary nucleotides into the sequencing primers of the nucleotide-complexed polymerases; h) detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases; and i) identifying the nucleo-bases of the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

[0075] In some embodiments, the method further comprises: forming at least one avidity complex in step (b), the method comprising: a) binding a first sequencing primer, a first sequencing polymerase, and a first multivalent molecule to a first portion of a nucleic acid template molecule thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first sequencing polymerase; and b) binding a second sequencing primer, a second sequencing polymerase, and the first multivalent molecule to a

second portion of the same nucleic acid template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the second multivalent molecule binds to the second sequencing polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms an avidity complex.

[0076] In some embodiments, (i) the first sequencing primer comprises a soluble forward sequencing primer and the nucleic acid template molecule comprises an immobilized concatemer template molecule, (ii) the second sequencing primer comprises a soluble forward sequencing primer and the nucleic acid template molecule comprises the same immobilized concatemer template molecule, and (iii) the first and second sequencing primers have the same sequence. In some embodiments, (i) the first sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises an immobilized partially displaced forward extension strand, (ii) the second sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises the same immobilized partially displaced forward extension strand, and (iii) the first and second sequencing primers have the same sequence. In some embodiments, (i) the first sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises an immobilized detached extended forward sequencing primer strand, (ii) the second sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises the same immobilized detached extended forward sequencing primer strand, and (iii) the first and second sequencing primers have the same sequence.

[0077] In some embodiments, the method further comprises: forming at least one avidity complex in step (b), the method comprising: a) contacting a plurality of first sequencing polymerases and a plurality of second sequencing primers with different portions of a nucleic acid template molecule to form at least first and second complexed polymerases on the same nucleic acid template molecule; b) contacting a plurality of multivalent molecules to the at least first and second complexed polymerases on the same nucleic acid template molecule, under conditions suitable to bind a single multivalent molecule from the plurality to the first and second complexed polymerases, wherein at least a first nucleotide unit of the single multivalent molecule is bound to the first complexed polymerase which includes a first sequencing primer hybridized to a first portion of the nucleic acid template molecule thereby forming a first binding complex, and wherein at least a second nucleotide unit of the single multivalent molecule is

bound to the second complexed polymerase which includes a second sequencing primer hybridized to a second portion of the same nucleic acid template molecule thereby forming a second binding complex, wherein the contacting is conducted under a condition suitable to inhibit polymerase-catalyzed incorporation of the bound first and second nucleotide units in the first and second binding complexes, and wherein the first and second binding complexes which are bound to the same multivalent molecule forms an avidity complex; c) detecting the first and second binding complexes on the same nucleic acid template molecule, and d) identifying the first nucleotide unit in the first binding complex thereby determining the sequence of the first portion of the nucleic acid template molecule, and identifying the second nucleotide unit in the second binding complex thereby determining the sequence of the second portion of the same nucleic acid template molecule.

[0078] In some embodiments, (i) the plurality of first sequencing primers comprise a plurality of first soluble forward sequencing primers and the nucleic acid template molecule comprises an immobilized concatemer template molecule, (ii) the plurality of second sequencing primers comprise a plurality of second soluble forward sequencing primers and the nucleic acid template molecule comprises the same immobilized concatemer template molecule, and (iii) the plurality of first and second sequencing primers have the same sequence. In some embodiments, (i) the plurality of first sequencing primers comprises a plurality of first soluble reverse sequencing primer and the nucleic acid template molecule comprises an immobilized partially displaced forward extension strand, (ii) the plurality of second sequencing primers comprise a plurality of second soluble reverse sequencing primers and the nucleic acid template molecule comprises the same immobilized partially displaced forward extension strand, and (iii) the plurality of first and second sequencing primers have the same sequence. In some embodiments, (i) the plurality of first sequencing primers comprises a plurality of first soluble reverse sequencing primer and the nucleic acid template molecule comprises an immobilized detached extended forward sequencing primer strands, (ii) the plurality of second sequencing primers comprise a plurality of second soluble reverse sequencing primers and the nucleic acid template molecule comprises the same immobilized detached extended forward sequencing primer strands, and (iii) the plurality of first and second sequencing primers have the same sequence.

[0079] In any of the foregoing or related embodiments, individual nucleotides in the plurality of nucleotides comprise an aromatic base, a five carbon sugar, and 1-10 phosphate groups,

wherein the aromatic base of the nucleotide comprises adenine, guanine, cytosine, thymine or uracil. In some embodiments, the plurality of nucleotides comprises one type of nucleotide selected from a group consisting of dATP, dGTP, dCTP and dTTP. In some embodiments, the plurality of nucleotides comprises a mixture of any combination of two or more types of nucleotides selected from a group consisting of dATP, dGTP, dCTP and/or dTTP. In some embodiments, at least one of the nucleotides in the plurality of nucleotides comprises a fluorescently-labeled nucleotide. In some embodiments, at least one of the plurality of nucleotides lacks a fluorophore label.

[0080] In any of the foregoing or related embodiments, at least one of the nucleotides in the plurality of nucleotides comprises a chain terminating moiety attached to 3'-OH sugar position via cleavable moiety, and wherein the chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group.

[0081] In some embodiments, the chain terminating moieties alkyl, alkenyl, alkynyl and allyl are cleavable/removable with tetrakis(triphenylphosphine)palladium(0) (Pd(PPh3)4) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ); (i) the chain terminating moieties aryl and benzyl are cleavable/removable with H2 Pd/C; (ii) the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable/removable with a thiol reagent which comprises beta-mercaptoethanol or dithiothritol (DTT); (iii) the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable/removable with a phosphine reagent which comprises Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP), or Tri(hydroxyproyl)phosphine (THPP); (iv) the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable/removable with 4-dimethylaminopyridine (4-DMAP); (v) the chain terminating moiety carbonate is cleavable/removable with potassium carbonate (K2CO3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH); and (vi) the chain terminating moieties urea and silyl are cleavable with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

[0082] In some embodiments, at least one of the nucleotides in the plurality of nucleotides comprises a chain terminating moiety attached to 3'-OH sugar position via cleavable moiety, and

wherein the chain terminating moiety comprises a 3' O-azido or a 3' O-azidomethyl group. In some embodiments, (i) the chain terminating moieties 3' O-azido and 3' O-azidomethyl group are cleavable/removable with a phosphine compound which comprise a derivatized tri-alkyl phosphine moiety, derivatized tri-aryl phosphine moiety, Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP); and (ii) the chain terminating moieties 3' O-azido and 3' O-azidomethyl group are cleavable/removable with 4-dimethylaminopyridine (4-DMAP).

[0083] In any of the foregoing or related embodiments, individual multivalent molecules in the plurality of multivalent molecules comprises (a) a core; and (b) a plurality of nucleotide arms which comprise (i) a core attachment moiety, (ii) a spacer comprising a PEG moiety, (iii) a linker, and (iv) a nucleotide unit, wherein the core is attached to the plurality of nucleotide arms via their core attachment moiety, wherein the spacer is attached to the linker, and wherein the linker is attached to the nucleotide unit.

[0084] In some embodiments, the core comprises an avidin-type moiety and the core attachment moiety comprises biotin. In some embodiments, the linker comprises an aliphatic chain having 2-6 subunits or an oligo ethylene glycol chain having 2-6 subunits. In some embodiments, the linker further comprises an aromatic moiety. In some embodiments, the nucleotide unit comprises an aromatic base, a five carbon sugar and 1-10 phosphate groups. In some embodiments, the linker is attached to the nucleotide unit through the base.

[0085] In some embodiments, the plurality of nucleotide arms attached to the core have the same type of a nucleotide unit, and wherein the types of nucleotide unit is selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP. In some embodiments, the plurality of multivalent molecules comprise one type of a multivalent molecule wherein each multivalent molecule in the plurality has the same type of nucleotide unit selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP. In some embodiments, the plurality of multivalent molecules comprise a mixture of any combination of two or more types of multivalent molecules each type having nucleotide units selected from a group consisting of dATP, dGTP, dCTP, dTTP and/or dUTP.

[0086] In some embodiments, the plurality of multivalent molecules are fluorescently-labeled multivalent molecules. In some embodiments, (i) the core of individual fluorescently-labeled multivalent molecules is attached to a fluorophore which corresponds to the nucleotide

units that are attached to the nucleotide arms; (ii) at least one of the nucleotide arms comprises a linker that is attached to a fluorophore which corresponds to the nucleotide units that are attached to the nucleotide arms; and/or (iii) at least one of the nucleotide arms comprises a nucleotide unit that is attached to a fluorophore which corresponds to the nucleotide units that are attached to the nucleotide arms.

[0087] In some embodiments, the plurality of multivalent molecules lack a fluorophore.

[0088] In some embodiments, at least one of the multivalent molecules in the plurality of multivalent molecules comprises nucleotide units having a chain terminating moiety attached to the 3'-OH sugar position via a cleavable moiety, and wherein the chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group.

[0089] In some embodiments, (i) the chain terminating moieties alkyl, alkenyl, alkynyl and allyl are cleavable/removable with tetrakis(triphenylphosphine)palladium(0) (Pd(PPh3)4) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ); (ii) the chain terminating moieties aryl and benzyl are cleavable/removable with H2 Pd/C; (iii) the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable/removable with a thiol reagent which comprises beta-mercaptoethanol or dithiothritol (DTT); (iv) the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable/removable with a phosphine reagent which comprises Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP), or Tri(hydroxyproyl)phosphine (THPP); (v) the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable/removable with 4-dimethylaminopyridine (4-DMAP); (vi) the chain terminating moiety carbonate is cleavable/removable with potassium carbonate (K2CO3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH); and (vii) the chain terminating moieties urea and silyl are cleavable with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

[0090] In some embodiments, at least one of the multivalent molecules in the plurality of multivalent molecules comprises nucleotide units having a chain terminating moiety attached to the 3'-OH sugar position via a cleavable moiety, and wherein the chain terminating moiety comprises a 3' O-azido or 3' O-azidomethyl group.

[0091] In some embodiments, (i) the chain terminating moieties 3' O-azido and 3' O-azidomethyl group are cleavable/removable with a phosphine compound which comprise a derivatized tri-alkyl phosphine moiety, derivatized tri-aryl phosphine moiety, Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP); and (ii) the chain terminating moieties 3' O-azido and 3' O-azidomethyl are cleavable/removable with 4-dimethylaminopyridine (4-DMAP).

[0092] In some embodiments, the plurality of sequencing polymerases in step (a) comprises a recombinant wild type DNA polymerase, and the plurality of nucleotides in step (b) comprises fluorescently-labeled nucleotides having a removable chain terminating moiety at the 3' sugar position.

[0093] In some embodiments, the plurality of sequencing polymerases in step (a) comprises a mutant DNA polymerase, and the plurality of nucleotides in step (b) comprises fluorescently-labeled nucleotides having a removable chain terminating moiety at the 3' sugar position.

[0094] In some embodiments, the plurality of first sequencing polymerases of step (a) comprise a recombinant wild type DNA polymerase. In some embodiments, the plurality of first sequencing polymerases of step (a) comprise mutant DNA polymerase.

[0095] In some embodiments, the plurality of second sequencing polymerases of step (f) comprise recombinant wild type DNA polymerase, and the plurality of nucleotides in step (b) comprises fluorescently-labeled nucleotides having a removable chain terminating moiety at the 3' sugar position.

[0096] In some embodiments, the plurality of second sequencing polymerases of step (f) comprise mutant DNA polymerase, and the plurality of nucleotides in step (b) comprises fluorescently-labeled nucleotides having a removable chain terminating moiety at the 3' sugar position.

[0097] In any of the foregoing or related embodiments, the replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction comprises: (i) contacting at least one extended forward sequencing primer strand with a plurality of strand displacing polymerases and a plurality of nucleotides and in the absence of soluble amplification primers, under a condition suitable to conduct a strand displacing primer extension reaction using the at least one extended

forward sequencing primers strand to initiate the primer extension reaction thereby generating a forward extension strand that is covalently joined to the extended forward sequencing primers strand, wherein the forward extension strand is hybridized to the immobilized concatemer template molecule.

[0098] In any of the foregoing or related embodiments, the replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction comprises removing the plurality of extended forward sequencing primer strands by: (i) contacting the plurality of extended forward sequencing primer strands with a 5' to 3' double-stranded DNA exonuclease; (ii) contacting the plurality of extended forward sequencing primer strands with a denaturation reagent comprising any combination of formamide, acetonitrile, guanidinium chloride and/or a pH buffering agent; or (iii) contacting the plurality of extended forward sequencing primer strands with 100% formamide.

[0099] In any of the foregoing or related embodiments, the replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction comprises: (i) removing the plurality of extended forward sequencing primer strands while retaining the immobilized concatemer template molecules; and (ii) contacting the plurality of retained immobilized concatemer molecules with a second plurality of soluble forward sequencing primers, a plurality of nucleotides and a plurality of primer extension polymerases, under a condition suitable to hybridize the plurality of soluble forward sequencing primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed primer extension reactions thereby generating a plurality of forward extension strands, wherein the plurality of nucleotides comprise dATP, dGTP, dCTP and dTTP but lacks dUTP, wherein in the plurality of primer extension polymerases are tolerant of uridine-containing template strands, and wherein the soluble sequencing primers hybridize with the forward sequencing primer binding sequence in the retained immobilized concatemer molecules.

[00100] In some embodiments, the contacting comprises: contacting the plurality of retained immobilized concatemer molecules with the plurality of soluble forward sequencing primers in

the presence of a high efficiency hybridization buffer which comprises: (i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer; (iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

[00101] In any of the foregoing or related embodiments, the replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction comprises: (i) removing the plurality of extended forward sequencing primer strand while retaining the immobilized concatemer template molecules; and (ii) contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble amplification primers, a plurality of nucleotides and a plurality of primer extension polymerases, under a condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed primer extension reactions thereby generating a plurality of forward extension strands, wherein the soluble amplification primers hybridize with the soluble amplification primer binding sequence in the retained immobilized concatemer molecules, wherein the plurality of nucleotides comprise dATP, dGTP, dCTP and dTTP but lacks dUTP, wherein in the plurality of primer extension polymerases are tolerant of uridine-containing template strands, and wherein the soluble sequencing primers hybridize with the forward sequencing primer binding sequence in the retained immobilized concatemer molecules.

[00102] In some embodiments, the contacting comprises: contacting the plurality of retained immobilized concatemer molecules with the plurality of soluble amplification primers in the presence of a high efficiency hybridization buffer which comprises: (i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer; (iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

[00103] In some embodiments, the method further comprises: contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble compaction oligonucleotides.

[00104] In any of the foregoing or related embodiments, the replacing the plurality of extended forward sequencing primer strands comprises: (i) contacting at least one extended forward sequencing primer strand with a plurality of strand displacing polymerases and a plurality of nucleotides and in the absence of soluble amplification primers, under a condition suitable to conduct a strand displacing primer extension reaction using the at least one extended forward sequencing primer strand to initiate the primer extension reaction thereby generating a plurality of forward extension strands, a plurality of partially displaced extended forward sequencing strands and a plurality of detached extended forward sequencing primer strands.

[00105] In any of the foregoing or related embodiments, replacing the plurality of extended forward sequencing primer strands comprises: comprises removing the plurality of extended forward sequencing primer strands by: (i) contacting the plurality of extended forward sequencing primer strands with a 5' to 3' double-stranded DNA exonuclease; (ii) contacting the plurality of extended forward sequencing primer strands with a denaturation reagent comprising any combination of formamide, acetonitrile, guanidinium chloride and/or a pH buffering agent; or (iii) contacting the plurality of extended forward sequencing primer strands with 100% formamide.

[00106] In any of the foregoing or related embodiments, the replacing the plurality of extended forward sequencing primer strands comprises: (i) removing the plurality of extended forward sequencing primer strands while retaining the immobilized concatemer template molecules; and (ii) contacting the plurality of retained immobilized concatemer molecules with a second plurality of soluble forward sequencing primers, a plurality of nucleotides and a plurality of strand displacing polymerases, under a condition suitable to hybridize the plurality of soluble forward sequencing primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed strand displacing reactions thereby generating a plurality of forward extension strands and a plurality of partially displaced extended forward sequencing strands that are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized amplicons, and the primer extension reaction generates a plurality of detached extended forward sequencing primer strands (e.g., that are not

hybridized to the immobilized concatemer template molecules), wherein the plurality of nucleotides comprise dATP, dGTP, dCTP and dTTP but lacks dUTP, and wherein the soluble forward sequencing primers hybridize with the forward sequencing primer binding sequence in the retained immobilized concatemer molecules.

[00107] In some embodiments, the contacting comprises: contacting the plurality of retained immobilized concatemer molecules with the plurality of soluble forward sequencing primers in the presence of a high efficiency hybridization buffer which comprises: (i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer; (iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

[00108] In any of the foregoing or related embodiments, the replacing the plurality of extended forward sequencing primer strands comprises: (i) removing the plurality of extended forward sequencing primer strand while retaining the immobilized concatemer template molecules; and (ii) contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble amplification primers, a plurality of nucleotides and a plurality of strand displacing polymerases, under a condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed strand displacing reactions thereby generating a plurality of forward extension strands and a plurality of partially displaced extended forward sequencing strands that are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized amplicons, and the primer extension reaction generates a plurality of detached extended forward sequencing primer strands (e.g., that are not hybridized to the immobilized concatemer template molecules), wherein the plurality of nucleotides comprise dATP, dGTP, dCTP and dTTP but lacks dUTP, wherein the soluble amplification primers hybridize with the soluble amplification primer binding sequence in the retained immobilized concatemer molecules.

[00109] In some embodiments, the contacting comprises: contacting the plurality of retained immobilized concatemer molecules with the plurality of soluble amplification primers in the presence of a high efficiency hybridization buffer which comprises: (i) a first polar aprotic

solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer; (iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

[00110] In any of the foregoing or related embodiments, the at least one of the retained immobilized concatemer template molecules includes one or more nucleotides having a scissile moiety, and wherein the scissile moiety comprises uridine or 8-oxo-7,8-dihydroguanine, or deoxyinosine. In any of the foregoing or related embodiments, the retained immobilized concatemer template molecule comprises one or more uridines, and wherein the generating the abasic sites at the uridines comprises contacting the retained immobilized concatemer template molecule with uracil DNA glycosylase (UDG). In any of the foregoing or related embodiments, the retained immobilized concatemer template molecule comprises one or more 8oxoG, and wherein the generating the abasic sites at the 8oxoG comprises contacting the retained immobilized concatemer template molecule with an Fpg enzyme (formamidopyrimidine DNA glycosylase). In any of the foregoing or related embodiments, the retained immobilized concatemer template molecule comprises one or more deoxyinosine, and wherein the generating the abasic sites at the deoxyinosine comprises contacting the retained immobilized concatemer template molecule with an AlkA glycosylase enzyme.

[00111] In any of the foregoing or related embodiments, the method further comprises generating a gap at the abasic sites to generate at least one gap-containing concatemer template molecule, which comprises: contacting the retained immobilized template molecules containing one or more abasic sites with an endonuclease IV, AP lyase (e.g., DNA-apurinic lyase or DNA-apyrimidinic lyase), FPG glycosylase/AP lyase and/or endo VIII glycosylase/AP lyase.

[00112] In any of the foregoing or related embodiments, the immobilized concatemer template molecules comprise 0.1 – 30% uridine, and wherein the plurality of wild type sequencing polymerases yield an error rate of incorporating dUTP of at least 0.1X compared to an error rate of incorporating dTTP. In any of the foregoing or related embodiments, the immobilized concatemer template molecules comprise 0.1 – 30% uridine, and wherein the plurality of mutant sequencing polymerases yield an error rate of incorporating dUTP of at least 0.1X compared to an error rate of incorporating dTTP. In any of the foregoing or related embodiments, the

immobilized concatemer template molecules comprise 0.1 – 30% uridine, and wherein the plurality of wild type sequencing polymerases yield an error rate of incorporating dUTP of at least 0.1X compared to an error rate of incorporating dTTP. In any of the foregoing or related embodiments, the immobilized concatemer template molecules comprise 0.1 – 30% uridine, and wherein the plurality of mutant sequencing polymerases yield an error rate of incorporating dUTP of at least 0.1X compared to an error rate of incorporating dTTP.

[00113] In any of the foregoing or related embodiments, the ratio of a first base fluorescent signal of R2 (e.g., reverse sequencing) to a first base fluorescent signal of R1 (e.g., forward sequencing) is at least 0.7 for sequencing using 1, 2, 3 or 4 dyes colors.

[00114] In any of the foregoing or related embodiments, the rolling circle amplification step comprises a plurality of compaction oligonucleotides and/or hexamine to generate immobilized concatemer template molecules having a more compact size and/or shape compared to a rolling circle amplification reaction in the absence of compaction oligonucleotides and/or hexamine.

[00115] In any of the foregoing or related embodiments, the primer extension reaction of step comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of forward extension strands having a more compact size and/or shape compared to a primer extension reaction in the absence of compaction oligonucleotides and/or hexamine.

[00116] In any of the foregoing or related embodiments, the rolling circle amplification step comprises a plurality of compaction oligonucleotides and/or hexamine to generate concatemer molecules having a more compact size and/or shape compared to a rolling circle amplification reaction in the absence of compaction oligonucleotides and/or hexamine.

[00117] In any of the foregoing or related embodiments, the primer extension reaction step comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of forward extension strands having a more compact size and/or shape compared to a primer extension reaction in the absence of compaction oligonucleotides and/or hexamine.

[00118] In any of the foregoing or related embodiments, the rolling circle amplification step comprises a plurality of compaction oligonucleotides and/or hexamine to generate immobilized concatemer template molecules having a more compact size and/or shape compared to a rolling circle amplification reaction in the absence of compaction oligonucleotides and/or hexamine.

[00119] In any of the foregoing or related embodiments, the primer extension reaction step comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of

forward extension strands having a more compact size and/or shape compared to a primer extension reaction in the absence of compaction oligonucleotides and/or hexamine.

[00120] In any of the foregoing or related embodiments, the primer extension reaction step comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of primer extension products having a more compact size and/or shape compared to a primer extension reaction in the absence of compaction oligonucleotides and/or hexamine, wherein the plurality of primer extension products include a plurality of forward extension strands, a plurality of partially displaced extended forward sequencing strands and a plurality of detached extended forward sequencing primer strands.

[00121] In any of the foregoing or related embodiments, the rolling circle amplification step comprises a plurality of compaction oligonucleotides and/or hexamine to generate immobilized concatemer template molecules having a more compact size and/or shape compared to a rolling circle amplification reaction in the absence of compaction oligonucleotides and/or hexamine.

[00122] In any of the foregoing or related embodiments, the primer extension reaction step comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of primer extension products having a more compact size and/or shape compared to a primer extension reaction in the absence of compaction oligonucleotides and/or hexamine, wherein the plurality of primer extension products include a plurality of forward extension strands, a plurality of partially displaced extended forward sequencing strands and a plurality of detached extended forward sequencing primer strands.

[00123] In any of the foregoing or related embodiments, the rolling circle amplification step comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of concatemer molecules having a more compact size and/or shape compared to a rolling circle amplification reaction in the absence of compaction oligonucleotides and/or hexamine.

[00124] In any of the foregoing or related embodiments, the primer extension reaction step comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of primer extension products having a more compact size and/or shape compared to a primer extension reaction in the absence of compaction oligonucleotides and/or hexamine, wherein the plurality of primer extension products include a plurality of forward extension strands, a plurality of partially displaced extended forward sequencing strands and a plurality of detached extended forward sequencing primer strands.

[00125] In any of the foregoing or related embodiments, the plurality of immobilized concatemer template molecules or the plurality of immobilized concatemer molecules have FWHM (full width half maximum) of no more than about 5 μm. In any of the foregoing or related embodiments, the plurality of forward extension strand have FWHM (full width half maximum) of no more than about 5 μm. In any of the foregoing or related embodiments, the plurality of primer extension products have FWHM (full width half maximum) of no more than about 5 μm.

DESCRIPTION OF THE DRAWINGS

[00126] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[00127] **Figure 1** is a schematic showing an exemplary single stranded nucleic acid concatemer template molecule immobilized to an immobilized first surface primer. The immobilized concatemer template molecule comprises at least one nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the immobilized concatemer template molecule. In some embodiments, the immobilized concatemer template molecule can be generated by conducting an on-support rolling circle amplification reaction. The arrangement of the various primer binding sequences is for illustration purposes. The skilled artisan will appreciate that many other arrangements are possible. Figures 2-12 show the workflow of pairwise sequencing the immobilized concatemer template molecule depicted in Figure 1.

[00128] **Figure 2** is a schematic showing an exemplary forward sequencing reaction conducted on the immobilized concatemer template molecule shown in Figure 1. The forward sequencing reaction can be conducted with a plurality of soluble forward sequencing primers and generates a plurality of extended forward sequencing primer strands. The immobilized concatemer template molecule can have two or more extended forward sequencing primer strands hybridized thereon.

[00129] **Figure 3** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a strand

displacing polymerase in the absence of a soluble primer thereby generating a forward extension strand.

[00130] **Figure 4** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a soluble forward sequencing primer thereby generating a forward extension strand.

[00131] **Figure 5** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a soluble amplification primer thereby generating a forward extension strand.

[00132] **Figure 6** is a schematic showing an exemplary method for generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotides having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers. The forward extension strand can be generated by the method depicted in Figures 3 or 4.

[00133] **Figure 7** is a schematic showing an exemplary retained forward extension strand after removal of the gap-containing concatemer template molecule as shown in Figure 6.

[00134] **Figure 8** is a schematic showing an exemplary is a schematic showing an exemplary method for generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotides having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers. The forward extension strand can be generated by the method depicted in Figure 5.

[00135] **Figure 9** is a schematic showing an exemplary retained forward extension strand after removal of the gap-containing concatemer template molecule as shown in Figure 8.

[00136] **Figure 10** is a schematic showing an exemplary reverse sequencing reaction conducted on the retained forward extension strand shown in Figure 7. The reverse sequencing reaction can be conducted with a plurality of soluble reverse sequencing primers. The retained forward extension strand can have two or more extended reverse sequencing primer strands hybridized thereon. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support. For the sake of simplicity,

Figures 1-10 show an exemplary immobilized concatemer molecule with one copy of the sequence of interest and various universal primer binding sites. The skilled artisan will appreciate that the immobilized concatemer molecule can include two or more tandem copies containing the sequence of interest and various universal primer binding sites.

[00137] **Figure 11** is a schematic showing an exemplary reverse sequencing reaction conducted on the retained forward extension strand shown in Figure 9. The retained forward extension strand can have two or more extended reverse sequencing primer strands hybridized thereon. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support. For the sake of simplicity, Figures 1-11 show an exemplary immobilized concatemer molecule with one copy of the sequence of interest and various universal primer binding sites. The skilled artisan will appreciate that the immobilized concatemer molecule can include two or more tandem copies containing the sequence of interest and various universal primer binding sites.

[00138] **Figure 12** is a schematic showing an exemplary support having a first and second surface primers immobilized thereon. A portion of the immobilized concatemer template molecule shown in Figure 1 is hybridized to the immobilized second surface primer. The immobilized concatemer template molecule has two or more copies of a universal binding sequence for an immobilized second surface primer. The portion of the immobilized concatemer template molecule that includes the universal binding sequence for an immobilized second surface primer can hybridize to the immobilized second surface primer.

[00139] **Figure 13** is a schematic showing an exemplary single stranded nucleic acid concatemer template molecule immobilized to an immobilized first surface primer. The immobilized concatemer template molecule comprises at least one nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the immobilized concatemer template molecule. In some embodiments, the immobilized concatemer template molecule can be generated by conducting an in-solution rolling circle amplification reaction and distributing the rolling circle amplification reaction onto the support. The arrangement of the various primer binding sequences is for illustration purposes. The skilled artisan will appreciate that many other arrangements are possible. Figures 14-25 show the workflow of pairwise sequencing the immobilized concatemer template molecule depicted in Figure 13.

[00140] **Figure 14** is a schematic showing an exemplary forward sequencing reaction conducted on the immobilized concatemer template molecule shown in Figure 13. The forward sequencing reaction can be conducted with a plurality of soluble forward sequencing primers. The immobilized concatemer template molecule can have two or more extended forward sequencing primer strands hybridized thereon.

[00141] **Figure 15** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a strand displacing polymerase in the absence of a soluble primer.

[00142] **Figure 16** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a soluble forward sequencing primer.

[00143] **Figure 17** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a soluble amplification primer.

[00144] **Figure 18** is a schematic showing an exemplary method for generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotides having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers. The forward extension strand can be generated by the method depicted in Figures 15 or 16.

[00145] **Figure 19** is a schematic showing an exemplary retained forward extension strand after removal of the gap-containing concatemer template molecule as shown in Figure 18.

[00146] **Figure 20** is a schematic showing an exemplary method for generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotides having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers. The forward extension strand can be generated by the method depicted in Figure 17.

[00147] **Figure 21** is a schematic showing an exemplary retained forward extension strand after removal of the gap-containing concatemer template molecule as shown in Figure 20.

[00148] **Figure 22** is a schematic showing an exemplary reverse sequencing reaction conducted on the retained forward extension strand shown in Figure 19. The reverse sequencing reaction can be conducted with a plurality of soluble reverse sequencing primers. The retained forward extension strand depicted in Figure 22 is a concatemer molecule that can include two or more tandem copies of the sequence of interest and various primer binding sites. Such a concatemer molecule can have two or more extended reverse sequencing primer strands hybridized thereon. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support. For the sake of simplicity, Figures 13-23 show an exemplary immobilized concatemer molecule with one copy of the sequence of interest and various universal primer binding sites. The skilled artisan will appreciate that the immobilized concatemer molecule can include two or more tandem copies containing the sequence of interest and various universal primer binding sites.

[00149] **Figure 23** is a schematic showing an exemplary reverse sequencing reaction conducted on the retained forward extension strand shown in Figure 21. The retained forward extension strand can have two or more extended reverse sequencing primer strands hybridized thereon. The retained forward extension strand depicted in Figure 23 is a concatemer molecule that includes two or more tandem copies of the sequence of interest and various primer binding sites. Such a concatemer molecule can have two or more extended reverse sequencing primer strands hybridized thereon. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support. For the sake of simplicity, Figures 13-23 show an exemplary immobilized concatemer molecule with two tandem copies containing the sequence of interest and various universal primer binding sites. The skilled artisan will appreciate that the immobilized concatemer molecule can include three or more tandem copies containing the sequence of interest and various universal primer binding sites.

[00150] **Figure 24** is a schematic showing an exemplary support having a first and second surface primers immobilized thereon. A portion of the immobilized concatemer template molecule shown in Figure 13 is hybridized to the immobilized second surface primer. The immobilized concatemer template molecule has two or more copies of a universal binding sequence for an immobilized second surface primer. The portion of the immobilized concatemer

template molecule that includes the universal binding sequence for an immobilized second surface primer can hybridize to the immobilized second surface primer.

[00151] **Figure 25** is a schematic showing an exemplary support having a first surface primer immobilized thereon, which in some embodiments, can be used to conduct an on-support pairwise sequencing workflow.

[00152] **Figure 26** is a schematic showing an exemplary on-support rolling circle amplification reaction using a nucleic acid circular library molecule, the immobilized first surface primer shown in Figure 25, and a mixture of nucleotides including nucleotides having a scissile moiety that can be cleaved to generate an abasic site. The rolling circle amplification reaction generates an immobilized single stranded nucleic acid concatemer template molecule having at least one nucleotide with a scissile moiety which can be cleaved to generate an abasic site in the immobilized concatemer template molecule. The arrangement of the various primer binding sequences in the nucleic acid circular library molecule is for illustration purposes. The skilled artisan will appreciate that many other arrangements are possible. Figures 26-37 show the workflow of pairwise sequencing the immobilized concatemer template molecule depicted in Figure 26.

[00153] **Figure 27** is a schematic showing an exemplary forward sequencing reaction conducted on the immobilized concatemer template molecule shown in Figure 26. The forward sequencing reaction can be conducted with a plurality of soluble forward sequencing primers and generates a plurality of extended forward sequencing primer strands. The immobilized concatemer template molecule can have two or more extended forward sequencing primer strands hybridized thereon.

[00154] **Figure 28** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a strand displacing polymerase in the absence of a soluble primer thereby generating a forward extension strand.

[00155] **Figure 29** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a soluble forward sequencing primer thereby generating a forward extension strand.

[00156] **Figure 30** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a soluble amplification primer thereby generating a forward extension strand.

[00157] **Figure 31** is a schematic showing an exemplary method for generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotides having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers. The forward extension strand can be generated by the method depicted in Figures 28 or 29.

[00158] **Figure 32** is a schematic showing an exemplary retained forward extension strand after removal of the gap-containing concatemer template molecule as shown in Figure 31.

[00159] **Figure 33** is a schematic showing an exemplary method for generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotides having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers. The forward extension strand can be generated by the method depicted in Figure 30.

[00160] **Figure 34** is a schematic showing an exemplary retained forward extension strand after removal of the gap-containing concatemer template molecule as shown in Figure 33.

[00161] **Figure 35** is a schematic showing an exemplary reverse sequencing reaction conducted on the retained forward extension strand shown in Figure 32. The reverse sequencing reaction can be conducted with a plurality of soluble reverse sequencing primers. The retained forward extension strand can have two or more extended reverse sequencing primer strands hybridized thereon. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support. For the sake of simplicity, Figures 26-36 show an exemplary immobilized concatemer molecule with one copy of the sequence of interest and various universal primer binding sites. The skilled artisan will appreciate that the immobilized concatemer molecule can include two or more tandem copies containing the sequence of interest and various universal primer binding sites.

[00162] **Figure 36** is a schematic showing an exemplary reverse sequencing reaction conducted on the retained forward extension strand shown in Figure 34. The retained forward extension strand can have two or more extended reverse sequencing primer strands hybridized thereon. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support. For the sake of simplicity, Figures 26-36 show an exemplary immobilized concatemer molecule with one copy of the sequence of interest and various universal primer binding sites. The skilled artisan will appreciate that the immobilized concatemer molecule can include two or more tandem copies containing the sequence of interest and various universal primer binding sites.

[00163] **Figure 37** is a schematic showing an exemplary support having a first and second surface primers immobilized thereon. A portion of the immobilized concatemer template molecule shown in Figure 26 is hybridized to the immobilized second surface primer. The immobilized concatemer template molecule has two or more copies of a universal binding sequence for an immobilized second surface primer. The portion of the immobilized concatemer template molecule that includes the universal binding sequence for an immobilized second surface primer can hybridize to the immobilized second surface primer.

[00164] **Figure 38** is a schematic showing an exemplary in-solution rolling circle amplification reaction using a nucleic acid circular library molecule, a soluble first amplification primer, and a mixture of nucleotides including nucleotides having a scissile moiety that can be cleaved to generate an abasic site. The rolling circle amplification reaction generates in solution single stranded nucleic acid concatemer molecules having at least one nucleotide with a scissile moiety which can be cleaved to generate an abasic site in the concatemer molecule. The arrangement of the various primer binding sequences in the nucleic acid circular library molecule is for illustration purposes. The skilled artisan will appreciate that many other arrangements are possible. Figures 38-52 show the workflow of pairwise sequencing the concatemer molecule depicted in Figure 38.

[00165] **Figure 39** is a schematic showing an exemplary method comprising distributing the rolling circle amplification reaction depicted in Figure 38 onto a support having a first surface primer immobilized thereon. The concatemer molecule can hybridize to the immobilized first surface primer.

[00166] **Figure 40** is a schematic showing an exemplary method which depicts the rolling circle amplification reaction continuing on the support thereby generating an immobilized concatemer template molecule which includes at least one nucleotide with a scissile moiety which can be cleaved to generate an abasic site in the immobilized concatemer template molecule.

[00167] **Figure 41** is a schematic showing an exemplary immobilized concatemer template molecule generated by the method depicted in Figure 40.

[00168] **Figure 42** is a schematic showing an exemplary forward sequencing reaction conducted on the immobilized concatemer template molecule shown in Figure 41. The forward sequencing reaction can be conducted with a plurality of soluble forward sequencing primers. The immobilized concatemer template molecule can have two or more extended forward sequencing primer strands hybridized thereon.

[00169] **Figure 43** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a strand displacing polymerase in the absence of a soluble primer.

[00170] **Figure 44** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a soluble forward sequencing primer.

[00171] **Figure 45** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a soluble amplification primer.

[00172] **Figure 46** is a schematic showing an exemplary method for generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotides having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers. The forward extension strand can be generated by the method depicted in Figure 43 or 44.

[00173] **Figure 47** is a schematic showing an exemplary retained forward extension strand after removal of the gap-containing concatemer template molecule as shown in Figure 46.

[00174] **Figure 48** is a schematic showing an exemplary method for generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotides having the

scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers. The forward extension strand can be generated by the method depicted in Figure 45.

[00175] **Figure 49** is a schematic showing an exemplary retained forward extension strand after removal of the gap-containing concatemer template molecule as shown in Figure 48.

[00176] **Figure 50** is a schematic showing an exemplary reverse sequencing reaction conducted on the retained forward extension strand shown in Figure 47. The reverse sequencing reaction can be conducted with a plurality of soluble reverse sequencing primers. The retained forward extension strand depicted in Figure 50 is a concatemer molecule that can include two or more tandem copies of the sequence of interest and various primer binding sites. Such a concatemer molecule can have two or more extended reverse sequencing primer strands hybridized thereon. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support. For the sake of simplicity, Figures 41-50 show an exemplary immobilized concatemer molecule with one copy of the sequence of interest and various universal primer binding sites. The skilled artisan will appreciate that the immobilized concatemer molecule can include two or more tandem copies containing the sequence of interest and various universal primer binding sites.

[00177] **Figure 51** is a schematic showing an exemplary reverse sequencing reaction conducted on the retained forward extension strand shown in Figure 49. The retained forward extension strand can have two or more extended reverse sequencing primer strands hybridized thereon. The retained forward extension strand depicted in Figure 51 is a concatemer molecule that includes two or more tandem copies of the sequence of interest and various primer binding sites. Such a concatemer molecule can have two or more extended reverse sequencing primer strands hybridized thereon. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support. For the sake of simplicity, Figures 41-51 show an exemplary immobilized concatemer molecule with two tandem copies containing the sequence of interest and various universal primer binding sites. The skilled artisan

will appreciate that the immobilized concatemer molecule can include three or more tandem copies containing the sequence of interest and various universal primer binding sites.

[00178] **Figure 52** is a schematic showing an exemplary support having a first and second surface primers immobilized thereon. A portion of the immobilized concatemer template molecule shown in Figure 41 is hybridized to the immobilized second surface primer. The immobilized concatemer template molecule has two or more copies of a universal binding sequence for an immobilized second surface primer. The portion of the immobilized concatemer template molecule that includes the universal binding sequence for an immobilized second surface primer can hybridize to the immobilized second surface primer.

[00179] **Figure 53** is schematic showing a linear single stranded library molecule (left top schematic) hybridizing with a double stranded splint molecule (left bottom schematic) to generate a circular library molecule with two gaps (right schematic). The splint molecule comprises a first splint strand (long strand) hybridized to a second splint strand (short strand). The first splint strand comprises a left sequence that hybridizes with a sequence on one end of the linear single stranded library molecule, and a right sequence that hybridizes with a sequence on the other end of the linear single stranded library molecule. The interior portion of the first splint strand hybridizes to the second splint strand.

[00180] **Figure 54** is a schematic showing the circular library molecule (left schematic) which is shown in Figure 53 undergoing a ligation reaction to generate a single stranded covalently closed circular molecule which is hybridized to the first splint strand (center schematic). The single stranded covalently closed circular molecule is subjected to a rolling circle amplification reaction using the 3' end of the first splint strand to initiate the RCA reaction (right schematic).

[00181] **Figure 55** is a schematic showing an exemplary support having a first surface primer immobilized thereon, which in some embodiments, can be used to conduct an on-support ligation reaction for a pairwise sequencing workflow. Figures 55-72 show the workflow of on-support ligation and pairwise sequencing.

[00182] **Figure 56** is a schematic showing an exemplary single stranded linear library molecule comprising a sequence of interest and various universal adaptor sequences for primer binding sites. The arrangement of the various universal adaptor sequences in this schematic is for illustration purposes. The skilled artisan will appreciate that many other arrangements, and combinations of universal adaptor sequences, are possible.

[00183] **Figure 57** is a schematic showing an exemplary single stranded linear library molecule hybridized to an immobilized first surface primer to form a circularized library molecule having an asymmetrically positioned gap or nick.

[00184] **Figure 58 (left)** is a schematic showing an exemplary single stranded linear library molecule hybridized to an immobilized first surface primer to form a circularized library molecule having an asymmetrically positioned gap or nick. **Figure 58 (right)** is a schematic showing an exemplary single stranded linear library molecule hybridized to an immobilized first surface primer to form a circularized library molecule having a symmetrically positioned gap or nick. The schematics shown in Figures 57 and 58 represent several embodiments of a circularized library molecule comprising a single stranded linear library molecule hybridized to an immobilized first surface primer.

[00185] **Figure 59** is a schematic showing an exemplary covalently closed circular library molecule generated by covalently closing the gap or nick.

[00186] **Figure 60 (left)** is a schematic showing an exemplary covalently closed circular library molecule generated by covalently closing the gap or nick. **Figure 60 (right)** is a schematic showing an exemplary covalently closed circular library molecule generated by covalently closing the gap or nick. The schematics shown in Figures 57 and 58 represent several embodiments of a covalently closed circular library molecule hybridized to an immobilized first surface primer.

[00187] **Figure 61** is a schematic showing an exemplary on-support rolling circle amplification reaction using a covalently closed circular library molecule, the immobilized first surface primer shown in Figure 55, and a mixture of nucleotides including nucleotides having a scissile moiety that can be cleaved to generate an abasic site. The rolling circle amplification reaction generates an immobilized single stranded nucleic acid concatemer template molecule having at least one nucleotide with a scissile moiety which can be cleaved to generate an abasic site in the immobilized concatemer template molecule.

[00188] **Figure 62** is a schematic showing an exemplary forward sequencing reaction conducted on the immobilized concatemer template molecule shown in Figure 61. The forward sequencing reaction can be conducted with a plurality of soluble forward sequencing primers and generates a plurality of extended forward sequencing primer strands. The immobilized

concatemer template molecule can have two or more extended forward sequencing primer strands hybridized thereon.

[00189] **Figure 63** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a strand displacing polymerase in the absence of a soluble primer thereby generating a forward extension strand.

[00190] **Figure 64** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a soluble forward sequencing primer thereby generating a forward extension strand.

[00191] **Figure 65** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with a soluble amplification primer thereby generating a forward extension strand.

[00192] **Figure 66** is a schematic showing an exemplary method for generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotides having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers. The forward extension strand can be generated by the method depicted in Figures 63 or 64.

[00193] **Figure 67** is a schematic showing an exemplary retained forward extension strand after removal of the gap-containing concatemer template molecule as shown in Figure 66.

[00194] **Figure 68** is a schematic showing an exemplary method for generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotides having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers. The forward extension strand can be generated by the method depicted in Figure 65.

[00195] **Figure 69** is a schematic showing an exemplary retained forward extension strand after removal of the gap-containing concatemer template molecule as shown in Figure 68.

[00196] **Figure 70** is a schematic showing an exemplary reverse sequencing reaction conducted on the retained forward extension strand shown in Figure 67. The reverse sequencing reaction can be conducted with a plurality of soluble reverse sequencing primers. The retained

forward extension strand can have two or more extended reverse sequencing primer strands hybridized thereon. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support.

[00197] **Figure 71** is a schematic showing an exemplary reverse sequencing reaction conducted on the retained forward extension strand shown in Figure 69. The retained forward extension strand can have two or more extended reverse sequencing primer strands hybridized thereon. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support.

[00198] **Figure 72** is a schematic showing an exemplary support having a first and second surface primers immobilized thereon. A portion of the immobilized concatemer template molecule shown in Figure 61 is hybridized to the immobilized second surface primer. The immobilized concatemer template molecule has two or more copies of a universal binding sequence for an immobilized second surface primer. The portion of the immobilized concatemer template molecule that includes the universal binding sequence for an immobilized second surface primer can hybridize to the immobilized second surface primer.

[00199] **Figure 73** is a schematic showing an exemplary single stranded nucleic acid concatemer template molecule immobilized to an immobilized first surface primer. In some embodiments, the immobilized concatemer template molecule can be generated by conducting an on-support rolling circle amplification reaction. The arrangement of the various primer binding sequences is for illustration purposes. The skilled artisan will appreciate that many other arrangements are possible. Figures 73-79 show the workflow of pairwise sequencing the immobilized concatemer template molecule depicted in Figure 73.

[00200] **Figure 74** is a schematic showing an exemplary forward sequencing reaction conducted on the immobilized concatemer template molecule shown in Figure 73. The forward sequencing reaction can be conducted with a plurality of soluble forward sequencing primers and generates a plurality of extended forward sequencing primer strands. The immobilized concatemer template molecule can have two or more extended forward sequencing primer strands hybridized thereon.

[00201] **Figure 75** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with soluble amplification primers and strand displacing polymerases in the presence of compaction oligonucleotides, thereby generating a forward extension strand and a partially displaced forward extension strand which are hybridized to the immobilized concatemer template molecule thereby forming an immobilized amplicon.

[00202] **Figure 76** is a schematic showing a continuation of the exemplary strand displacing method shown in Figure 75, where the polymerase-catalyzed strand displacing reaction generates a forward extension strand and a partially displaced forward extension strand which are hybridized to the immobilized concatemer template molecule, and a detached forward extension strand which is not hybridized to the immobilized concatemer template molecule.

[00203] **Figure 77** is a schematic showing an exemplary hybridization complex comprising a forward extension strand and a partially displaced forward extension strand which are hybridized to the immobilized concatemer template molecule, and an immobilized detached forward extension strand which is hybridized to the partially displaced forward extension strand.

[00204] **Figure 78** is a schematic showing an exemplary reverse sequencing reaction conducted on the hybridization complex shown in Figure 77. The reverse sequencing reaction can be conducted with a plurality of soluble reverse sequencing primers on the partially displaced forward extension strand and the immobilized detached forward extension strand. The reverse sequencing reaction generates extended reverse sequencing primer strands. For the sake of simplicity, Figure 78 shows one copy of an extended reverse sequencing primer strand on the partially displaced forward extension strand, and one copy of an extended reverse sequencing primer strand on the immobilized detached forward extension strand. The skilled artisan will appreciate that the partially displaced forward extension strand and the immobilized detached forward extension strand can include two or more extended reverse sequencing primer strands hybridized thereon.

[00205] **Figure 79** is a schematic showing an exemplary support having a first and second surface primers immobilized thereon. A portion of the immobilized concatemer template molecule shown in Figure 73 is hybridized to the immobilized second surface primer. The immobilized concatemer template molecule has two or more copies of a universal binding sequence for an immobilized second surface primer. The portion of the immobilized concatemer

template molecule that includes the universal binding sequence for an immobilized second surface primer can hybridize to the immobilized second surface primer.

[00206] **Figure 80** is a schematic showing an exemplary single stranded nucleic acid concatemer template molecule immobilized to an immobilized first surface primer. In some embodiments, the immobilized concatemer template molecule can be generated by conducting an in-solution rolling circle amplification reaction and distributing the rolling circle amplification reaction onto the support. The arrangement of the various primer binding sequences is for illustration purposes. The skilled artisan will appreciate that many other arrangements are possible. Figures 80-86 show the workflow of pairwise sequencing the immobilized concatemer template molecule depicted in Figure 80.

[00207] **Figure 81** is a schematic showing an exemplary forward sequencing reaction conducted on the immobilized concatemer template molecule shown in Figure 80. The forward sequencing reaction can be conducted with a plurality of soluble forward sequencing primers. The immobilized concatemer template molecule can have two or more extended forward sequencing primer strands hybridized thereon.

[00208] **Figure 82** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with soluble amplification primers and strand displacing polymerases in the presence of compaction oligonucleotides, thereby generating a forward extension strand and a partially displaced forward extension strand which are hybridized to the immobilized concatemer template molecule thereby forming an immobilized amplicon.

[00209] **Figure 83** is a schematic showing a continuation of the exemplary strand displacing method shown in Figure 82, where the polymerase-catalyzed strand displacing reaction generates a forward extension strand and a partially displaced forward extension strand which are hybridized to the immobilized concatemer template molecule, and a detached forward extension strand which is not hybridized to the immobilized concatemer template molecule.

[00210] **Figure 84** is a schematic showing an exemplary hybridization complex comprising a forward extension strand and a partially displaced forward extension strand which are hybridized to the immobilized concatemer template molecule, and an immobilized detached forward extension strand which is hybridized to the partially displaced forward extension strand.

[00211] **Figure 85** is a schematic showing an exemplary reverse sequencing reaction conducted on the hybridization complex shown in Figure 84. The reverse sequencing reaction can be conducted with a plurality of soluble reverse sequencing primers on the partially displaced forward extension strand and the immobilized detached forward extension strand. The reverse sequencing reaction generates extended reverse sequencing primer strands. For the sake of simplicity, Figure 85 shows one copy of an extended reverse sequencing primer strand on the partially displaced forward extension strand, and one copy of an extended reverse sequencing primer strand on the immobilized detached forward extension strand. The skilled artisan will appreciate that the partially displaced forward extension strand and the immobilized detached forward extension strand can include two or more extended reverse sequencing primer strands hybridized thereon.

[00212] **Figure 86** is a schematic showing an exemplary support having a first and second surface primers immobilized thereon. A portion of the immobilized concatemer template molecule shown in Figure 80 is hybridized to the immobilized second surface primer. The immobilized concatemer template molecule has two or more copies of a universal binding sequence for an immobilized second surface primer. The portion of the immobilized concatemer template molecule that includes the universal binding sequence for an immobilized second surface primer can hybridize to the immobilized second surface primer.

[00213] **Figure 87** is a schematic showing an exemplary support having a first surface primer immobilized thereon, which in some embodiments, can be used to conduct an on-support pairwise sequencing workflow.

[00214] **Figure 88** is a schematic showing an exemplary on-support rolling circle amplification reaction using a nucleic acid circular library molecule, the immobilized first surface primer shown in Figure 87. The rolling circle amplification reaction generates an immobilized single stranded nucleic acid concatemer template molecule. The arrangement of the various primer binding sequences in the nucleic acid circular library molecule is for illustration purposes. The skilled artisan will appreciate that many other arrangements are possible. Figures 87-94 show the workflow of pairwise sequencing the immobilized concatemer template molecule depicted in Figure 87.

[00215] **Figure 89** is a schematic showing an exemplary single stranded nucleic acid concatemer template molecule immobilized to an immobilized first surface primer.

[00216] **Figure 90** is a schematic showing an exemplary forward sequencing reaction conducted on the immobilized concatemer template molecule shown in Figure 89. The forward sequencing reaction can be conducted with a plurality of soluble forward sequencing primers and generates a plurality of extended forward sequencing primer strands. The immobilized concatemer template molecule can have two or more extended forward sequencing primer strands hybridized thereon.

[00217] **Figure 91** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with soluble amplification primers and strand displacing polymerases in the presence of compaction oligonucleotides, thereby generating a forward extension strand and a partially displaced forward extension strand which are hybridized to the immobilized concatemer template molecule thereby forming an immobilized amplicon.

[00218] **Figure 92** is a schematic showing a continuation of the exemplary strand displacing method shown in Figure 91, where the polymerase-catalyzed strand displacing reaction generates a forward extension strand and a partially displaced forward extension strand which are hybridized to the immobilized concatemer template molecule, and a detached forward extension strand which is not hybridized to the immobilized concatemer template molecule.

[00219] **Figure 93** is a schematic showing an exemplary hybridization complex comprising a forward extension strand and a partially displaced forward extension strand which are hybridized to the immobilized concatemer template molecule, and an immobilized detached forward extension strand which is hybridized to the partially displaced forward extension strand.

[00220] **Figure 94** is a schematic showing an exemplary reverse sequencing reaction conducted on the hybridization complex shown in Figure 93. The reverse sequencing reaction can be conducted with a plurality of soluble reverse sequencing primers on the partially displaced forward extension strand and the immobilized detached forward extension strand. The reverse sequencing reaction generates extended reverse sequencing primer strands. For the sake of simplicity, Figure 94 shows one copy of an extended reverse sequencing primer strand on the partially displaced forward extension strand, and one copy of an extended reverse sequencing primer strand on the immobilized detached forward extension strand. The skilled artisan will appreciate that the partially displaced forward extension strand and the immobilized detached

forward extension strand can include two or more extended reverse sequencing primer strands hybridized thereon.

[00221] **Figure 95** is a schematic showing an exemplary in-solution rolling circle amplification reaction using a nucleic acid circular library molecule, a soluble first amplification primer, and a mixture of nucleotides. The rolling circle amplification reaction generates in solution single stranded nucleic acid concatemer molecules. The arrangement of the various primer binding sequences in the nucleic acid circular library molecule is for illustration purposes. The skilled artisan will appreciate that many other arrangements are possible. Figures 95-103 show the workflow of pairwise sequencing the concatemer molecule depicted in Figure 96.

[00222] **Figure 96** is a schematic showing an exemplary method comprising distributing the rolling circle amplification reaction depicted in Figure 95 onto a support having a first surface primer immobilized thereon. The concatemer molecule can hybridize to the immobilized first surface primer.

[00223] **Figure 97** is a schematic showing an exemplary method which depicts the rolling circle amplification reaction continuing on the support thereby generating an immobilized concatemer template molecule.

[00224] **Figure 98** is a schematic showing an exemplary single stranded nucleic acid concatemer template molecule immobilized to an immobilized first surface primer.

[00225] **Figure 99** is a schematic showing an exemplary forward sequencing reaction conducted on the immobilized concatemer template molecule shown in Figure 98. The forward sequencing reaction can be conducted with a plurality of soluble forward sequencing primers. The immobilized concatemer template molecule can have two or more extended forward sequencing primer strands hybridized thereon.

[00226] **Figure 100** is a schematic showing an exemplary method for replacing the extended forward sequencing primer strands by conducting a primer extension reaction with soluble amplification primers and strand displacing polymerases in the presence of compaction oligonucleotides, thereby generating a forward extension strand and a partially displaced forward extension strand which are hybridized to the immobilized concatemer template molecule thereby forming an immobilized amplicon.

[00227] **Figure 101** is a schematic showing a continuation of the exemplary strand displacing method shown in Figure 100, where the polymerase-catalyzed strand displacing reaction

generates a forward extension strand and a partially displaced forward extension strand which are hybridized to the immobilized concatemer template molecule, and a detached forward extension strand which is not hybridized to the immobilized concatemer template molecule.

[00228] **Figure 102** is a schematic showing an exemplary hybridization complex comprising a forward extension strand and a partially displaced forward extension strand which are hybridized to the immobilized concatemer template molecule, and an immobilized detached forward extension strand which is hybridized to the partially displaced forward extension strand.

[00229] **Figure 103** is a schematic showing an exemplary reverse sequencing reaction conducted on the hybridization complex shown in Figure 102. The reverse sequencing reaction can be conducted with a plurality of soluble reverse sequencing primers on the partially displaced forward extension strand and the immobilized detached forward extension strand. The reverse sequencing reaction generates extended reverse sequencing primer strands. For the sake of simplicity, Figure 103 shows one copy of an extended reverse sequencing primer strand on the partially displaced forward extension strand, and one copy of an extended reverse sequencing primer strand on the immobilized detached forward extension strand. The skilled artisan will appreciate that the partially displaced forward extension strand and the immobilized detached forward extension strand can include two or more extended reverse sequencing primer strands hybridized thereon.

[00230] **Figure 104** is a schematic of various exemplary configurations of multivalent molecules. **Left:** schematics of multivalent molecules having a starburst or helter-skelter configuration. **Center:** a schematic of a multivalent molecule having a dendrimer configuration. **Right:** a schematic of multiple multivalent molecules formed by reacting streptavidin with 4-arm or 8-arm PEG-NHS with biotin and dNTPs. Nucleotide units are designated 'N', biotin is designated 'B', and streptavidin is designated 'SA'.

[00231] **Figure 105** is a schematic of an exemplary multivalent molecules comprising a generic core attached to a plurality of nucleotide-arms.

[00232] **Figure 106** is a schematic of an exemplary multivalent molecule comprising a dendrimer core attached to a plurality of nucleotide-arms.

[00233] **Figure 107** shows a schematic of an exemplary multivalent molecule comprising a core attached to a plurality of nucleotide-arms, where the nucleotide arms comprise biotin, spacer, linker and a nucleotide unit.

[00234] **Figure 108** is a schematic of an exemplary nucleotide-arm comprising a core attachment moiety, spacer, linker and nucleotide unit.

[00235] **Figure 109** shows the chemical structure of an exemplary spacer, and the chemical structures of various exemplary linkers, including an 11-atom Linker, 16-atom Linker, 23-atom Linker and an N3 Linker.

[00236] **Figure 110** shows the chemical structures of various exemplary linkers, including Linkers 1-9.

[00237] **Figure 111** shows the chemical structures of various exemplary linkers joined/attached to nucleotide units.

[00238] **Figure 112** shows the chemical structures of various exemplary linkers joined/attached to nucleotide units.

[00239] **Figure 113** shows the chemical structures of various exemplary linkers joined/attached to nucleotide units.

[00240] **Figure 114** shows the chemical structure of an exemplary nucleotide-arm. In this example, the nucleotide unit is connected to the linker via a propargyl amine attachment at the 5 position of a pyrimidine base or the 7 position of a purine base. This nucleotide-arm shows an exemplary biotinylated nucleotide-arm.

[00241] **Figure 115** is an exemplary schematic illustration of one embodiment of the low binding support comprising a glass substrate and alternating layers of hydrophilic coatings which are covalently or non-covalently adhered to the glass, and which further comprises chemically-reactive functional groups that serve as attachment sites for oligonucleotide primers (e.g., capture oligonucleotides and circularization oligonucleotides). In an alternative embodiment, the support can be made of any material such as glass, plastic or a polymer material.

[00242] **Figure 116A** is a schematic of a guanine tetrad (e.g., G-tetrad).

[00243] **Figure 116B** is a schematic of an intramolecular G-quadruplex structure.

[00244] **Figure 117** is a schematic of an exemplary single cycle showing flowing in a nucleic acid relaxing buffer with temperature ramp-up and ramp-down, a washing step, and flowing in a flexing amplification buffer containing a strand-displacing DNA polymerase with temperature ramp-up and MDA incubation and ramp-down. One or more cycles can be conducted of the flowing in a flexing amplification buffer containing a strand-displacing DNA polymerase with temperature ramp-up and MDA amplification and ramp-down.

[00245] **Figure 118** (left) is a graph showing the error rate from R1 sequencing reads of template molecules having various levels of uracil. **Figure 118** (right) is a graph showing the phasing rate from R1 sequencing reads of template molecules having various levels of uracil. The data shows that sequencing template molecules having lower levels of incorporated uracil yield lower error rates and phasing rates. The level of uracil in the template molecules also affects the intensity ratio of R2/R1 reads.

[00246] **Figure 119** is a graph showing increased ratio of signal intensity for R2/R1 sequencing reads when the sequencing workflow employs a cleaving reagent that includes a compound that reduces photo-damage to nucleic acids. Lanes 1, 3, 5 and 7 show the R2/R1 signal intensity using different cleaving reagent formulations without a compound that reduces photo-damage. Lanes 2, 4, 6 and 8 show the R2/R1 signal intensity using corresponding cleaving reagent formulations that include a compound that reduces photo-damage.

DETAILED DESCRIPTION

Definitions

[00247] The headings provided herein are not limitations of the various aspects of the disclosure, which aspects can be understood by reference to the specification as a whole.

[00248] Unless defined otherwise, technical and scientific terms used herein have meanings that are commonly understood by those of ordinary skill in the art unless defined otherwise. Generally, terminologies pertaining to techniques of molecular biology, nucleic acid chemistry, protein chemistry, genetics, microbiology, transgenic cell production, and hybridization described herein are those well-known and commonly used in the art. Techniques and procedures described herein are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the instant specification. For example, see Sambrook et al., Molecular Cloning: A Laboratory Manual (Third ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 2000). See also Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates (1992). The nomenclatures utilized in connection with, and the laboratory procedures and techniques described herein are those well-known and commonly used in the art.

[00249] Unless otherwise required by context herein, singular terms shall include pluralities and plural terms shall include the singular. Singular forms "a", "an" and "the", and singular use of any word, include plural referents unless expressly and unequivocally limited on one referent.

[00250] It is understood the use of the alternative term (e.g., "or") is taken to mean either one or both or any combination thereof of the alternatives.

[00251] The term "and/or" used herein is to be taken mean specific disclosure of each of the specified features or components with or without the other. For example, the term "and/or" as used in a phrase such as "A and/or B" herein is intended to include: "A and B"; "A or B"; "A" (A alone); and "B" (B alone). In a similar manner, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following aspects: "A, B, and C"; "A, B, or C"; "A or C"; "A or B"; "B or C"; "A and B"; "B and C"; "A and C"; "A" (A alone); "B" (B alone); and "C" (C alone).

[00252] As used herein and in the appended claims, terms "comprising", "including", "having" and "containing", and their grammatical variants, as used herein are intended to be non-limiting so that one item or multiple items in a list do not exclude other items that can be substituted or added to the listed items. It is understood that wherever aspects are described herein with the language "comprising," otherwise analogous aspects described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[00253] As used herein, the terms "about" and "approximately" refer to a value or composition that is within an acceptable error range for the particular value or composition as determined by one of ordinary skill in the art, which will depend in part on how the value or composition is measured or determined, i.e., the limitations of the measurement system. For example, "about" or "approximately" can mean within one or more than one standard deviation per the practice in the art. Alternatively, "about" or "approximately" can mean a range of up to 10% (i.e., ±10%) or more depending on the limitations of the measurement system. For example, about 5 mg can include any number between 4.5 mg and 5.5 mg. Furthermore, particularly with respect to biological systems or processes, the terms can mean up to an order of magnitude or up to 5-fold of a value. When particular values or compositions are provided in the instant disclosure, unless otherwise stated, the meaning of "about" or "approximately" should be assumed to be within an acceptable error range for that particular value or composition. Also,

where ranges and/or subranges of values are provided, the ranges and/or subranges can include the endpoints of the ranges and/or subranges.

[00254] The term "biological sample" refers to a single cell, a plurality of cells, a tissue, an organ, an organism, or section of any of these biological samples. The biological sample can be extracted (e.g., biopsied) from an organism, or obtained from a cell culture grown in liquid or in a culture dish. The biological sample comprises a sample that is fresh, frozen, fresh frozen, or archived (e.g., formalin-fixed paraffin-embedded; FFPE). The biological sample can be embedded in a wax, resin, epoxy or agar. The biological sample can be fixed, for example in any one or any combination of two or more of acetone, ethanol, methanol, formaldehyde, paraformaldehyde-Triton or glutaraldehyde. The biological sample can be sectioned or non-sectioned. The biological sample can be stained, de-stained or non-stained.

[00255] The nucleic acids of interest can be extracted from biological samples using any of a number of techniques known to those of skill in the art. For example, a typical DNA extraction procedure comprises (i) collection of the cell sample or tissue sample from which DNA is to be extracted, (ii) disruption of cell membranes (i.e., cell lysis) to release DNA and other cytoplasmic components, (iii) treatment of the lysed sample with a concentrated salt solution to precipitate proteins, lipids, and RNA, followed by centrifugation to separate out the precipitated proteins, lipids, and RNA, and (iv) purification of DNA from the supernatant to remove detergents, proteins, salts, or other reagents used during the cell membrane lysis. A variety of suitable commercial nucleic acid extraction and purification kits are consistent with the disclosure herein. Examples include, but are not limited to, the QIAamp kits (for isolation of genomic DNA from human samples) and DNAeasy kits (for isolation of genomic DNA from animal or plant samples) from Qiagen (Germantown, MD), or the Maxwell® and ReliaPrep™ series of kits from Promega (Madison, WI).

[00256] The terms "nucleic acid", "polynucleotide" and "oligonucleotide" and other related terms used herein are used interchangeably and refer to polymers of nucleotides and are not limited to any particular length. Nucleic acids include recombinant and chemically-synthesized forms. Nucleic acids can be isolated. Nucleic acids include DNA molecules (e.g., cDNA or genomic DNA), RNA molecules (e.g., mRNA), analogs of the DNA or RNA generated using nucleotide analogs (e.g., peptide nucleic acids (PNA) and non-naturally occurring nucleotide analogs), and chimeric forms containing DNA and RNA. Nucleic acids can be single-stranded

or double-stranded. Nucleic acids comprise polymers of nucleotides, where the nucleotides include natural or non-natural bases and/or sugars. Nucleic acids comprise naturally-occurring internucleosidic linkages, for example phosphdiester linkages. Nucleic acids can lack a phosphate group. Nucleic acids comprise non-natural internucleoside linkages, including phosphorothioate, phosphorothiolate, or peptide nucleic acid (PNA) linkages. In some embodiments, nucleic acids comprise a one type of polynucleotides or a mixture of two or more different types of polynucleotides.

[00257] The term "universal sequence", "universal adaptor sequences" and related terms refers to a sequence in a nucleic acid molecule that is common among two or more polynucleotide molecules. For example, adaptors having the same universal sequence can be joined to a plurality of polynucleotides so that the population of co-joined molecules carry the same universal adaptor sequence. Examples of universal adaptor sequences include an amplification primer sequence, a sequencing primer sequence or a capture primer sequence (e.g., soluble or support-immobilized capture primers).

[00258] The term "operably linked" and "operably joined" or related terms as used herein refers to juxtaposition of components. The juxtapositioned components can be linked together covalently. For example, two nucleic acid components can be enzymatically ligated together where the linkage that joins together the two components comprises phosphodiester linkage. A first and second nucleic acid component can be linked together, where the first nucleic acid component can confer a function on a second nucleic acid component. For example, linkage between a primer binding sequence and a sequence of interest forms a nucleic acid library molecule having a portion that can bind to a primer. In another example, a transgene (e.g., a nucleic acid encoding a polypeptide or a nucleic acid sequence of interest) can be ligated to a vector where the linkage permits expression or functioning of the transgene sequence contained in the vector. In some embodiments, a transgene is operably linked to a host cell regulatory sequence (e.g., a promoter sequence) that affects expression of the transgene. In some embodiments, the vector comprises at least one host cell regulatory sequence, including a promoter sequence, enhancer, transcription and/or translation initiation sequence, transcription and/or translation termination sequence, polypeptide secretion signal sequences, and the like. In some embodiments, the host cell regulatory sequence controls expression of the level, timing and/or location of the transgene.

[00259] The terms "linked", "joined", "attached", "appended" and variants thereof comprise any type of fusion, bond, adherence or association between any combination of compounds or molecules that is of sufficient stability to withstand use in the particular procedure. The procedure can include but are not limited to: nucleotide binding; nucleotide incorporation; de-blocking (e.g., removal of chain-terminating moiety); washing; removing; flowing; detecting; imaging and/or identifying. Such linkage can comprise, for example, covalent, ionic, hydrogen, dipole-dipole, hydrophilic, hydrophobic, or affinity bonding, bonds or associations involving van der Waals forces, mechanical bonding, and the like. In some embodiments, such linkage occurs intramolecularly, for example linking together the ends of a single-stranded or double-stranded linear nucleic acid molecule to form a circular molecule. In some embodiments, such linkage can occur between a combination of different molecules, or between a molecule and a non-molecule, including but not limited to: linkage between a nucleic acid molecule and a solid surface; linkage between a protein and a detectable reporter moiety; linkage between a nucleotide and detectable reporter moiety; and the like. Some examples of linkages can be found, for example, in Hermanson, G., "Bioconjugate Techniques", Second Edition (2008); Aslam, M., Dent, A., "Bioconjugation: Protein Coupling Techniques for the Biomedical Sciences", London: Macmillan (1998); Aslam, M., Dent, A., "Bioconjugation: Protein Coupling Techniques for the Biomedical Sciences", London: Macmillan (1998).

[00260] The term "adaptor" and related terms refers to oligonucleotides that can be operably linked (appended) to a target polynucleotide, where the adaptor confers a function to the co-joined adaptor-target molecule. Adaptors comprise DNA, RNA, chimeric DNA/RNA, or analogs thereof. Adaptors can include at least one ribonucleoside residue. Adaptors can be single-stranded, double-stranded, or have single-stranded and/or double-stranded portions. Adaptors can be configured to be linear, stem-looped, hairpin, or Y-shaped forms. Adaptors can be any length, including 4-100 nucleotides or longer. Adaptors can have blunt ends, overhang ends, or a combination of both. Overhang ends include 5' overhang and 3' overhang ends. The 5' end of a single-stranded adaptor, or one strand of a double-stranded adaptor, can have a 5' phosphate group or lack a 5' phosphate group. Adaptors can include a 5' tail that does not hybridize to a target polynucleotide (e.g., tailed adaptor), or adaptors can be non-tailed. An adaptor can include a sequence that is complementary to at least a portion of a primer, such as an amplification primer, a sequencing primer, or a capture primer (e.g., soluble or immobilized capture primers).

Adaptors can include a random sequence or degenerate sequence. Adaptors can include at least one inosine residue. Adaptors can include at least one phosphorothioate, phosphorothiolate and/or phosphoramidate linkage. Adaptors can include a barcode sequence which can be used to distinguish polynucleotides (e.g., insert sequences) from different sample sources in a multiplex assay. Adaptors can include a unique identification sequence (e.g., unique molecular index, UMI; or a unique molecular tag) that can be used to uniquely identify a nucleic acid molecule to which the adaptor is appended. In some embodiments, a unique identification sequence can be used to increase error correction and accuracy, reduce the rate of false-positive variant calls and/or increase sensitivity of variant detection. Adaptors can include at least one restriction enzyme recognition sequence, including any one or any combination of two or more selected from a group consisting of type I, type II, type III, type IV, type Hs or type IIB.

[00261] The term "nucleic acid template", "template polynucleotide", "nucleic acid target" "target polynucleotide", "template strand" and other variations refer to a nucleic acid strand that serves as the basis nucleic acid molecule for any of the analysis methods describe herein (e.g., primer extension, amplifying and/or sequencing). The template nucleic acid can be single-stranded or double-stranded, or the template nucleic acid can have single-stranded or double-stranded portions. The template nucleic acid can be obtained from a naturally-occurring source, recombinant form, or chemically synthesized to include any type of nucleic acid analog. The template nucleic acid can be linear, circular, or other forms. The template nucleic acids can include an insert region having an insert sequence which is also known as a sequence of interest. The template nucleic acids can also include at least one adaptor sequence. The template nucleic acid can be a concatemer having two or tandem copies of a sequence of interest and at least one adaptor sequence. The insert region can be isolated in any form, including chromosomal, genomic, organellar (e.g., mitochondrial, chloroplast or ribosomal), recombinant molecules, cloned, amplified, cDNA, RNA such as precursor mRNA or mRNA, oligonucleotides, whole genomic DNA, obtained from fresh frozen paraffin embedded tissue, needle biopsies, circulating tumor cells, cell free circulating DNA, or any type of nucleic acid library. The insert region can be isolated from any source including from organisms such as prokaryotes, eukaryotes (e.g., humans, plants and animals), fungus, viruses cells, tissues, normal or diseased cells or tissues, body fluids including blood, urine, serum, lymph, tumor, saliva, anal and vaginal secretions, amniotic samples, perspiration, semen, environmental samples, culture samples, or synthesized

nucleic acid molecules prepared using recombinant molecular biology or chemical synthesis methods. The insert region can be isolated from any organ, including head, neck, brain, breast, ovary, cervix, colon, rectum, endometrium, gallbladder, intestines, bladder, prostate, testicles, liver, lung, kidney, esophagus, pancreas, thyroid, pituitary, thymus, skin, heart, larynx, or other organs. The template nucleic acid can be subjected to nucleic acid analysis, including sequencing and composition analysis.

[00262] The term "polymerase" and its variants, as used herein, comprises an enzyme comprising a domain that binds a nucleotide (or nucleoside) where the polymerase can form a complex having a template nucleic acid and a complementary nucleotide. The polymerase can have one or more activities including, but not limited to, base analog detection activities, DNA polymerization activity, reverse transcriptase activity, DNA binding, strand displacement activity, and nucleotide binding and recognition. A polymerase can be any enzyme that can catalyze 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. Typically, a polymerase comprises one or more active sites at which nucleotide binding and/or catalysis of nucleotide polymerization can occur. In some embodiments, a polymerase includes other enzymatic activities, such as for example, 3′ to 5′ exonuclease activity or 5′ to 3′ exonuclease activity. In some embodiments, a polymerase has strand displacing activity. A polymerase 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 nucleotide polymerization (e.g., catalytically active fragment). The polymerase includes catalytically inactive polymerases, catalytically active polymerases, reverse transcriptases, and other enzymes comprising a nucleotide binding domain. In some embodiments, a polymerase can be isolated from a cell, or generated using recombinant DNA technology or chemical synthesis methods. In some embodiments, a polymerase can be expressed in prokaryote, eukaryote, viral, or phage organisms. In some embodiments, a polymerase can be post-translationally modified proteins or fragments thereof. A polymerase can be derived from a prokaryote, eukaryote, virus or phage. A polymerase comprises DNA-directed DNA polymerase and RNA-directed DNA polymerase.

[00263] The term "strand displacing" refers to the ability of a polymerase to locally separate strands of double-stranded nucleic acids and synthesize a new strand in a template-based manner. Strand displacing polymerases displace a complementary strand from a template strand and catalyze new strand synthesis. Strand displacing polymerases include mesophilic and thermophilic polymerases. Strand displacing polymerases include wild type enzymes, and variants including exonuclease minus mutants, mutant versions, chimeric enzymes and truncated enzymes. Examples of strand displacing polymerases include phi29 DNA polymerase, large fragment of Bst DNA polymerase, large fragment of Bsu DNA polymerase (exo-), Bca DNA polymerase (exo-), Klenow fragment of E. coli DNA polymerase, T5 polymerase, M-MuLV reverse transcriptase, HIV viral reverse transcriptase, Deep Vent DNA polymerase and KOD DNA polymerase. The phi29 DNA polymerase can be wild type phi29 DNA polymerase (e.g., MagniPhi from Expedeon), or variant EquiPhi29 DNA polymerase (e.g., from Thermo Fisher Scientific), or chimeric QualiPhi DNA polymerase (e.g., from 4basebio).

[00264] As used herein, the term "DNA primase-polymerase" and related terms refers to enzymes having activities of a DNA polymerase and an RNA primase. A DNA primase-polymerase enzyme can utilize deoxyribonucleotide triphosphates to synthesize a DNA primer on a single-stranded DNA template in a template-sequence dependent manner, and can extend the primer strand via nucleotide polymerization (e.g., primer extension), in the presence of a catalytic divalent cation (e.g., magnesium and/or manganese). The DNA primase-polymerase include enzymes that are members of DnaG-like primases (e.g., bacteria) and AEP-like primases (Archaea and Eukaryotes). An exemplary DNA primase-polymerase enzyme is Tth PrimPol from *Thermus thermophilus* HB27.

[00265] As used herein, the term "fidelity" refers to the accuracy of DNA polymerization by template-dependent DNA polymerase. The fidelity of a DNA polymerase is typically measured by the error rate (the frequency of incorporating an inaccurate nucleotide, i.e., a nucleotide that is not complementary to the template nucleotide). The accuracy or fidelity of DNA polymerization is maintained by both the polymerase activity and the 3′-5′ exonuclease activity of a DNA polymerase.

[00266] As used herein, the term "binding complex" refers to a complex formed by binding together a nucleic acid duplex, a polymerase, and a free nucleotide or a nucleotide unit of a multivalent molecule, where the nucleic acid duplex comprises a nucleic acid template molecule

hybridized to a nucleic acid primer. In the binding complex, the free nucleotide or nucleotide unit may or may not be bound to the 3' end of the nucleic acid primer at a position that is opposite a complementary nucleotide in the nucleic acid template molecule. A "ternary complex" is an example of a binding complex which is formed by binding together a nucleic acid duplex, a polymerase, and a free nucleotide or nucleotide unit of a multivalent molecule, where the free nucleotide or nucleotide unit is bound to the 3' end of the nucleic acid primer (as part of the nucleic acid duplex) at a position that is opposite a complementary nucleotide in the nucleic acid template molecule.

[00267] The term "persistence time" and related terms refers to the length of time that a binding complex remains stable without dissociation of any of the components, where the components of the binding complex include a nucleic acid template and nucleic acid primer, a polymerase, a nucleotide unit of a multivalent molecule or a free (e.g., unconjugated) nucleotide. The nucleotide unit or the free nucleotide can be complementary or non-complementary to a nucleotide residue in the template molecule. The nucleotide unit or the free nucleotide can bind to the 3' end of the nucleic acid primer at a position that is opposite a complementary nucleotide residue in the nucleic acid template molecule. The persistence time is indicative of the stability of the binding complex and strength of the binding interactions. Persistence time can be measured by observing the onset and/or duration of a binding complex, such as by observing a signal from a labeled component of the binding complex. For example, a labeled nucleotide or a labeled reagent comprising one or more nucleotides may be present in a binding complex, thus allowing the signal from the label to be detected during the persistence time of the binding complex. One exemplary label is a fluorescent label. The binding complex (e.g., ternary complex) remains stable until subjected to a condition that causes dissociation of interactions between any of the polymerase, template molecule, primer and/or the nucleotide unit or the nucleotide. For example, a dissociating condition comprises contacting the binding complex with any one or any combination of a detergent, EDTA and/or water.

[00268] The term "primer" and related terms used herein refers to an oligonucleotide that is capable of hybridizing with a DNA and/or RNA polynucleotide template to form a duplex molecule. Primers comprise natural nucleotides and/or nucleotide analogs. Primers can be recombinant nucleic acid molecules. Primers may have any length, but typically range from 4-50 nucleotides. A typical primer comprises a 5' end and 3' end. The 3' end of the primer can

include a 3' OH moiety which serves as a nucleotide polymerization initiation site in a polymerase-catalyzed primer extension reaction. Alternatively, the 3' end of the primer can lack a 3' OH moiety, or can include a terminal 3' blocking group that inhibits nucleotide polymerization in a polymerase-catalyzed reaction. Any one nucleotide, or more than one nucleotide, along the length of the primer can be labeled with a detectable reporter moiety. A primer can be in solution (e.g., a soluble primer) or can be immobilized to a support (e.g., a capture primer).

[00269] When used in reference to nucleic acid molecules, the terms "hybridize" or "hybridizing" or "hybridization" or other related terms refers to hydrogen bonding between two different nucleic acids to form a duplex nucleic acid. Hybridization also includes hydrogen bonding between two different regions of a single nucleic acid molecule to form a self-hybridizing molecule having a duplex region. Hybridization can comprise Watson-Crick or Hoogstein binding to form a duplex double-stranded nucleic acid, or a double-stranded region within a nucleic acid molecule. The double-stranded nucleic acid, or the two different regions of a single nucleic acid, may be wholly complementary, or partially complementary. Complementary nucleic acid strands need not hybridize with each other across their entire length. 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.

[00270] When used in reference to nucleic acids, the terms "extend", "extending", "extension" and other variants, refers to incorporation of one or more nucleotides into a nucleic acid molecule. Nucleotide incorporation comprises polymerization of one or more nucleotides into the terminal 3' OH end of a nucleic acid strand (e.g., a nucleic acid primer), resulting in extension of the nucleic acid strand (e.g., extended primer). Nucleotide incorporation can be conducted with natural nucleotides and/or nucleotide analogs. Typically, but not necessarily, nucleotide incorporation occurs in a template-dependent fashion. Any suitable method of extending a nucleic acid molecule may be used, including primer extension catalyzed by a DNA polymerase or RNA polymerase.

[00271] In some embodiments, any of the amplification primer sequences, sequencing primer sequences, capture primer sequences (capture oligonucleotides), target capture sequences, circularization anchor sequences, sample barcode sequences, spatial barcode sequences, or

anchor region sequences can be about 3-50 nucleotides in length, or about 5-40 nucleotides in length, or about 5-25 nucleotides in length.

[00272] The term "nucleotides" and related terms refers to a molecule comprising an aromatic base, a five carbon sugar (e.g., ribose or deoxyribose), and at least one phosphate group. Canonical or non-canonical nucleotides are consistent with use of the term. The phosphate in some embodiments comprises a monophosphate, diphosphate, or triphosphate, or corresponding phosphate analog. The term "nucleoside" refers to a molecule comprising an aromatic base and a sugar. Nucleotides and nucleosides can be non-labeled or labeled with a detectable reporter moiety.

[00273] Nucleotides (and nucleosides) typically comprise a hetero cyclic base including substituted or unsubstituted nitrogen-containing parent heteroaromatic ring which are commonly found in nucleic acids, including naturally-occurring, substituted, modified, or engineered variants, or analogs of the same. The base of a nucleotide (or nucleoside) is capable of forming Watson-Crick and/or Hoogstein hydrogen bonds with an appropriate complementary base. Exemplary bases include, but are not limited to, purines and pyrimidines such as: 2-aminopurine, 2,6-diaminopurine, adenine (A), ethenoadenine, N^6-Δ^2-isopentenyladenine (6iA), N^6-Δ^2-isopentenyl-2-methylthioadenine (2ms6iA), N^6-methyladenine, guanine (G), isoguanine, N^2-dimethylguanine (dmG), 7-methylguanine (7mG), 2-thiopyrimidine, 6-thioguanine (6sG), hypoxanthine and O^6-methylguanine; 7-deaza-purines such as 7-deazaadenine (7-deaza-A) and 7-deazaguanine (7-deaza-G); pyrimidines such as cytosine (C), 5-propynylcytosine, isocytosine, thymine (T), 4-thiothymine (4sT), 5,6-dihydrothymine, O^4-methylthymine, uracil (U), 4-thiouracil (4sU) and 5,6-dihydrouracil (dihydrouracil; D); indoles such as nitroindole and 4-methylindole; pyrroles such as nitropyrrole; nebularine; inosines; hydroxymethylcytosines; 5-methycytosines; base (Y); as well as methylated, glycosylated, and acylated base moieties; and the like. Additional exemplary bases can be found in Fasman, 1989, in "Practical Handbook of Biochemistry and Molecular Biology", pp. 385-394, CRC Press, Boca Raton, Fla.

[00274] Nucleotides (and nucleosides) typically comprise a sugar moiety, such as carbocyclic moiety (Ferraro and Gotor 2000 Chem. Rev. 100: 4319-48), acyclic moieties (Martinez, et al., 1999 Nucleic Acids Research 27: 1271-1274; Martinez, et al., 1997 Bioorganic & Medicinal Chemistry Letters vol. 7: 3013-3016), and other sugar moieties (Joeng, et al., 1993 J. Med. Chem. 36: 2627-2638; Kim, et al., 1993 J. Med. Chem. 36: 30-7; Eschenmosser 1999 Science

284:2118-2124; and U.S. Pat. No. 5,558,991). The sugar moiety comprises: ribosyl; 2′-deoxyribosyl; 3′-deoxyribosyl; 2′,3′-dideoxyribosyl; 2′,3′-didehydrodideoxyribosyl; 2′-alkoxyribosyl; 2′-azidoribosyl; 2′-aminoribosyl; 2′-fluororibosyl; 2′-mercaptoriboxyl; 2′-alkylthioribosyl; 3′-alkoxyribosyl; 3′-azidoribosyl; 3′-aminoribosyl; 3′-fluororibosyl; 3′-mercaptoriboxyl; 3′-alkylthioribosyl carbocyclic; acyclic or other modified sugars.

[00275] In some embodiments, nucleotides comprise a chain of one, two or three phosphorus atoms where the chain is typically attached to the 5' carbon of the sugar moiety via an ester or phosphoramide linkage. In some embodiments, the nucleotide is an analog having a phosphorus chain in which the phosphorus atoms are linked together with intervening O, S, NH, methylene or ethylene. In some embodiments, the phosphorus atoms in the chain include substituted side groups including O, S or BH_3. In some embodiments, the chain includes phosphate groups substituted with analogs including phosphoramidate, phosphorothioate, phosphordithioate, and O-methylphosphoroamidite groups.

[00276] The term "reporter moiety", "reporter moieties" or related terms refers to a compound that generates, or causes to generate, a detectable signal. A reporter moiety is sometimes called a "label". Any suitable reporter moiety may be used, including luminescent, photoluminescent, electroluminescent, bioluminescent, chemiluminescent, fluorescent, phosphorescent, chromophore, radioisotope, electrochemical, mass spectrometry, Raman, hapten, affinity tag, atom, or an enzyme. A reporter moiety generates a detectable signal resulting from a chemical or physical change (e.g., heat, light, electrical, pH, salt concentration, enzymatic activity, or proximity events). A proximity event includes two reporter moieties approaching each other, or associating with each other, or binding each other. It is well known to one skilled in the art to select reporter moieties so that each absorbs excitation radiation and/or emits fluorescence at a wavelength distinguishable from the other reporter moieties to permit monitoring the presence of different reporter moieties in the same reaction or in different reactions. Two or more different reporter moieties can be selected having spectrally distinct emission profiles, or having minimal overlapping spectral emission profiles. Reporter moieties can be linked (e.g., operably linked) to nucleotides, nucleosides, nucleic acids, enzymes (e.g., polymerases or reverse transcriptases), or support (e.g., surfaces).

[00277] A reporter moiety (or label) comprises a fluorescent label or a fluorophore. Exemplary fluorescent moieties which may serve as fluorescent labels or fluorophores include,

but are not limited to fluorescein and fluorescein derivatives such as carboxyfluorescein, tetrachlorofluorescein, hexachlorofluorescein, carboxynapthofluorescein, fluorescein isothiocyanate, NHS-fluorescein, iodoacetamidofluorescein, fluorescein maleimide, SAMSA-fluorescein, fluorescein thiosemicarbazide, carbohydrazinomethylthioacetyl-amino fluorescein, rhodamine and rhodamine derivatives such as TRITC, TMR, lissamine rhodamine, Texas Red, rhodamine B, rhodamine 6G, rhodamine 10, NHS-rhodamine, TMR-iodoacetamide, lissamine rhodamine B sulfonyl chloride, lissamine rhodamine B sulfonyl hydrazine, Texas Red sulfonyl chloride, Texas Red hydrazide, coumarin and coumarin derivatives such as AMCA, AMCA-NHS, AMCA-sulfo-NHS, AMCA-HPDP, DCIA, AMCE-hydrazide, BODIPY and derivatives such as BODIPY FL C3-SE, BODIPY 530/550 C3, BODIPY 530/550 C3-SE, BODIPY 530/550 C3 hydrazide, BODIPY 493/503 C3 hydrazide, BODIPY FL C3 hydrazide, BODIPY FL IA, BODIPY 530/551 IA, Br-BODIPY 493/503, Cascade Blue and derivatives such as Cascade Blue acetyl azide, Cascade Blue cadaverine, Cascade Blue ethylenediamine, Cascade Blue hydrazide, Lucifer Yellow and derivatives such as Lucifer Yellow iodoacetamide, Lucifer Yellow CH, cyanine and derivatives such as indolium based cyanine dyes, benzo-indolium based cyanine dyes, pyridium based cyanine dyes, thiozolium based cyanine dyes, quinolinium based cyanine dyes, imidazolium based cyanine dyes, Cy 3, Cy5, lanthanide chelates and derivatives such as BCPDA, TBP, TMT, BHHCT, BCOT, Europium chelates, Terbium chelates, Alexa Fluor dyes, DyLight dyes, Atto dyes, LightCycler Red dyes, CAL Flour dyes, JOE and derivatives thereof, Oregon Green dyes, WellRED dyes, IRD dyes, phycoerythrin and phycobilin dyes, Malachite green, stilbene, DEG dyes, NR dyes, near-infrared dyes and others known in the art such as those described in Haugland, Molecular Probes Handbook, (Eugene, Oreg.) 6th Edition; Lakowicz, Principles of Fluorescence Spectroscopy, 2nd Ed., Plenum Press New York (1999), or Hermanson, Bioconjugate Techniques, 2nd Edition, or derivatives thereof, or any combination thereof. Cyanine dyes may exist in either sulfonated or non-sulfonated forms, and consist of two indolenin, benzo-indolium, pyridium, thiozolium, and/or quinolinium groups separated by a polymethine bridge between two nitrogen atoms. Commercially available cyanine fluorophores include, for example, Cy3, (which may comprise 1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-2-(3-{1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-3,3-dimethyl-1,3-dihydro-2H-indol-2-ylidene}prop-1-en-1-yl)-3,3-dimethyl-3H-indolium or 1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-2-(3-{1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-3,3-dimethyl-5-sulfo-

1,3-dihydro-2H-indol-2-ylidene}prop-1-en-1-yl)-3,3-dimethyl-3H-indolium-5-sulfonate), Cy5 (which may comprise 1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-2-((1E,3E)-5-((E)-1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium or 1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-2-((1E,3E)-5-((E)-1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate), and Cy7 (which may comprise 1-(5-carboxypentyl)-2-[(1E,3E,5E,7Z)-7-(1-ethyl-1,3-dihydro-2H-indol-2-ylidene)hepta-1,3,5-trien-1-yl]-3H-indolium or 1-(5-carboxypentyl)-2-[(1E,3E,5E,7Z)-7-(1-ethyl-5-sulfo-1,3-dihydro-2H-indol-2-ylidene)hepta-1,3,5-trien-1-yl]-3H-indolium-5-sulfonate), where "Cy" stands for 'cyanine', and the first digit identifies the number of carbon atoms between two indolenine groups. Cy2 which is an oxazole derivative rather than indolenin, and the benzo-derivatized Cy3.5, Cy5.5 and Cy7.5 are exceptions to this rule.

[00278] In some embodiments, the reporter moiety can be a FRET pair, such that multiple classifications can be performed under a single excitation and imaging step. As used herein, FRET may comprise excitation exchange (Forster) transfers, or electron-exchange (Dexter) transfers.

[00279] The term "support" as used herein refers to a substrate that is designed for deposition of biological molecules or biological samples for assays and/or analyses. Examples of biological molecules to be deposited onto a support include nucleic acids (e.g., DNA, RNA), polypeptides, saccharides, lipids, a single cell or multiple cells. Examples of biological samples include but are not limited to saliva, phlegm, mucus, blood, plasma, serum, urine, stool, sweat, tears and fluids from tissues or organs.

[00280] In some embodiments, the support is solid, semi-solid, or a combination of both. In some embodiments, the support is porous, semi-porous, non-porous, or any combination of porosity. In some embodiments, the support can be substantially planar, concave, convex, or any combination thereof. In some embodiments, the support can be cylindrical, for example comprising a capillary or interior surface of a capillary.

[00281] In some embodiments, the surface of the support can be substantially smooth. In some embodiments, the support can be regularly or irregularly textured, including bumps, etched, pores, three-dimensional scaffolds, or any combination thereof.

[00282] In some embodiments, the support comprises a bead having any shape, including spherical, hemi-spherical, cylindrical, barrel-shaped, toroidal, disc-shaped, rod-like, conical, triangular, cubical, polygonal, tubular or wire-like.

[00283] The support can be fabricated from any material, including but not limited to glass, fused-silica, silicon, a polymer (e.g., polystyrene (PS), macroporous polystyrene (MPPS), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET)), or any combination thereof. Various compositions of both glass and plastic substrates are contemplated.

[00284] The support can have a plurality (e.g., two or more) of nucleic acid templates immobilized thereon. The plurality of immobilized nucleic acid templates have the same sequence or have different sequences. In some embodiments, individual nucleic acid template molecules in the plurality of nucleic acid templates are immobilized to a different site on the support. In some embodiments, two or more individual nucleic acid template molecules in the plurality of nucleic acid templates are immobilized to a site on the support.

[00285] The term "array" refers to a support comprising a plurality of sites located at pre-determined locations on the support to form an array of sites. The sites can be discrete and separated by interstitial regions. In some embodiments, the pre-determined sites on the support can be arranged in one dimension in a row or a column, or arranged in two dimensions in rows and columns. In some embodiments, the plurality of pre-determined sites is arranged on the support in an organized fashion. In some embodiments, the plurality of pre-determined sites is arranged in any organized pattern, including rectilinear, hexagonal patterns, grid patterns, patterns having reflective symmetry, patterns having rotational symmetry, or the like. The pitch between different pairs of sites can be that same or can vary. In some embodiments, the support comprises at least 10^2 sites, at least 10^3 sites, at least 10^4 sites, at least 10^5 sites, at least 10^6 sites, at least 10^7 sites, at least 10^8 sites, at least 10^9 sites, at least 10^{10} sites, at least 10^{11} sites, at least 10^{12} sites, at least 10^{13} sites, at least 10^{14} sites, at least 10^{15} sites, or more, where the sites are located at pre-determined locations on the support. In some embodiments, a plurality of pre-determined sites on the support (e.g., $10^2 - 10^{15}$ sites or more) are immobilized with nucleic acid templates to form a nucleic acid template array. In some embodiments, the nucleic acid templates that are immobilized at a plurality of pre-determined sites by hybridization to immobilized

surface capture primers, or the nucleic acid templates are covalently attached to the surface capture primer. In some embodiments, the nucleic acid templates that are immobilized at a plurality of pre-determined sites, for example immobilized at $10^2 - 10^{15}$ sites or more. In some embodiments, the immobilized nucleic acid templates are clonally-amplified to generate immobilized nucleic acid polonies at the plurality of pre-determined sites. In some embodiments, individual immobilized nucleic acid polonies comprise single-stranded or double-stranded concatemers.

[00286] In some embodiments, a support comprising a plurality of sites located at random locations on the support is referred to herein as a support having randomly located sites thereon. The location of the randomly located sites on the support are not pre-determined. The plurality of randomly-located sites is arranged on the support in a disordered and/or unpredictable fashion. In some embodiments, the support comprises at least 10^2 sites, at least 10^3 sites, at least 10^4 sites, at least 10^5 sites, at least 10^6 sites, at least 10^7 sites, at least 10^8 sites, at least 10^9 sites, at least 10^{10} sites, at least 10^{11} sites, at least 10^{12} sites, at least 10^{13} sites, at least 10^{14} sites, at least 10^{15} sites, or more, where the sites are randomly located on the support. In some embodiments, a plurality of randomly located sites on the support (e.g., $10^2 - 10^{15}$ sites or more) are immobilized with nucleic acid templates to form a support immobilized with nucleic acid templates. In some embodiments, the nucleic acid templates that are immobilized at a plurality of randomly located sites by hybridization to immobilized surface capture primers, or the nucleic acid templates are covalently attached to the surface capture primer. In some embodiments, the nucleic acid templates that are immobilized at a plurality of randomly located sites, for example immobilized at $10^2 - 10^{15}$ sites or more. In some embodiments, the immobilized nucleic acid templates are clonally-amplified to generate immobilized nucleic acid polonies at the plurality of randomly located sites. In some embodiments, individual immobilized nucleic acid polonies comprise single-stranded or double-stranded concatemers.

[00287] When used in reference to a low binding surface coating, one or more layers of a multi-layered surface coating may comprise a branched polymer or may be linear. Examples of suitable branched polymers include, but are not limited to, branched PEG, branched poly(vinyl alcohol) (branched PVA), branched poly(vinyl pyridine), branched poly(vinyl pyrrolidone) (branched PVP), branched), poly(acrylic acid) (branched PAA), branched polyacrylamide, branched poly(N-isopropylacrylamide) (branched PNIPAM), branched poly(methyl

methacrylate) (branched PMA), branched poly(2-hydroxylethyl methacrylate) (branched PHEMA), branched poly(oligo(ethylene glycol) methyl ether methacrylate) (branched POEGMA), branched polyglutamic acid (branched PGA), branched poly-lysine, branched poly-glucoside, and dextran.

[00288] In some embodiments, the branched polymers used to create one or more layers of any of the multi-layered surfaces disclosed herein may comprise at least 4 branches, at least 5 branches, at least 6 branches, at least 7 branches, at least 8 branches, at least 9 branches, at least 10 branches, at least 12 branches, at least 14 branches, at least 16 branches, at least 18 branches, at least 20 branches, at least 22 branches, at least 24 branches, at least 26 branches, at least 28 branches, at least 30 branches, at least 32 branches, at least 34 branches, at least 36 branches, at least 38 branches, or at least 40 branched.

[00289] Linear, branched, or multi-branched polymers used to create one or more layers of any of the multi-layered surfaces disclosed herein may have a molecular weight of 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, at least 15,000, at least 20,000, at least 25,000, at least 30,000, at least 35,000, at least 40,000, at least 45,000, or at least 50,000 daltons.

[00290] In some embodiments, e.g., wherein at least one layer of a multi-layered surface comprises a branched polymer, the number of covalent bonds between a branched polymer molecule of the layer being deposited and molecules of the previous layer may range from about one covalent linkage per molecule and about 32 covalent linkages per molecule. In some embodiments, the number of covalent bonds between a branched polymer molecule of the new layer and molecules of the previous layer may be at least 1, 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 14, at least 16, at least 18, at least 20, at least 22, at least 24, at least 26, at least 28, at least 30, or at least 32 covalent linkages per molecule.

[00291] Any reactive functional groups that remain following the coupling of a material layer to the surface may optionally be blocked by coupling a small, inert molecule using a high yield coupling chemistry. For example, in the case that amine coupling chemistry is used to attach a new material layer to the previous one, any residual amine groups may subsequently be acetylated or deactivated by coupling with a small amino acid such as glycine.

[00292] The number of layers of low non-specific binding material, e.g., a hydrophilic polymer material, deposited on the surface, may range from 1 to about 10. In some embodiments, the number of layers is at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10. In some embodiments, the number of layers may be at most 10, at most 9, at most 8, at most 7, at most 6, at most 5, at most 4, at most 3, at most 2, or at most 1. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some embodiments the number of layers may range from about 2 to about 4. In some embodiments, all of the layers may comprise the same material. In some embodiments, each layer may comprise a different material. In some embodiments, the plurality of layers may comprise a plurality of materials. In some embodiments at least one layer may comprise a branched polymer. In some embodiment, all of the layers may comprise a branched polymer.

[00293] One or more layers of low non-specific binding material may in some cases be deposited on and/or conjugated to the substrate surface using a polar protic solvent, a polar or polar aprotic solvent, a nonpolar solvent, or any combination thereof. In some embodiments the solvent used for layer deposition and/or coupling may comprise an alcohol (e.g., methanol, ethanol, propanol, etc.), another organic solvent (e.g., acetonitrile, dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), etc.), water, an aqueous buffer solution (e.g., phosphate buffer, phosphate buffered saline, 3-(N-morpholino)propanesulfonic acid (MOPS), etc.), or any combination thereof. In some embodiments, an organic component of the solvent mixture used may comprise at least 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% of the total, with the balance made up of water or an aqueous buffer solution. In some embodiments, an aqueous component of the solvent mixture used may comprise at least 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% of the total, with the balance made up of an organic solvent. The pH of the solvent mixture used may be less than 6, about 6, 6.5, 7, 7.5, 8, 8.5, 9, or greater than pH 9.

[00294] The term "branched polymer" and related terms refers to a polymer having a plurality of functional groups that help conjugate a biologically active molecule such as a nucleotide, and the functional group can be either on the side chain of the polymer or directly attaches to a central core or central backbone of the polymer. The branched polymer can have linear backbone

with one or more functional groups coming off the backbone for conjugation. The branched polymer can also be a polymer having one or more sidechains, wherein the side chain has a site suitable for conjugation. Examples of the functional group include but are limited to hydroxyl, ester, amine, carbonate, acetal, aldehyde, aldehyde hydrate, alkenyl, acrylate, methacrylate, acrylamide, active sulfone, hydrazide, thiol, alkanoic acid, acid halide, isocyanate, isothiocyanate, maleimide, vinylsulfone, dithiopyridine, vinylpyridine, iodoacetamide, epoxide, glyoxal, dione, mesylate, tosylate, and tresylate.

[00295] When used in reference to immobilized nucleic acids, the term "immobilized" and related terms refer to nucleic acid molecules that are attached to a support through covalent bond or non-covalent interaction, or attached to a coating on the support, or buried within a matrix formed by a coating on the support, where the nucleic acid molecules include surface capture primers, nucleic acid template molecules and extension products of capture primers. Extension products of capture primers includes nucleic acid concatemers that can form nucleic acid polonies.

[00296] In some embodiments, one or more nucleic acid templates are immobilized on the support, for example immobilized at the sites on the support. In some embodiments, the one or more nucleic acid templates are clonally-amplified. In some embodiments, the one or more nucleic acid templates are clonally-amplified off the support (e.g., in-solution) and then deposited onto the support and immobilized on the support. In some embodiments, the clonal amplification reaction of the one or more nucleic acid templates is conducted on the support resulting in immobilization on the support. In some embodiments, the one or more nucleic acid templates are clonally-amplified (e.g., in solution or on the support) using a nucleic acid amplification reaction, including any one or any combination of: polymerase chain reaction (PCR), multiple displacement amplification (MDA), transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), real-time SDA, bridge amplification, isothermal bridge amplification, rolling circle amplification (RCA), circle-to-circle amplification, helicase-dependent amplification, recombinase-dependent amplification, and/or single-stranded binding (SSB) protein-dependent amplification.

[00297] The term "surface primer", "surface capture primer" and related terms refers to single-stranded oligonucleotides that are immobilized to a support and comprise a sequence that

can hybridize to at least a portion of a nucleic acid template molecule. Surface primers can be used to immobilize template molecules to a support via hybridization. Surface primers can be immobilized to a support in a manner that resists primer removal during flowing, washing, aspirating, and changes in temperature, pH, salts, chemical and/or enzymatic conditions. Typically, but not necessarily, the 5' end of a surface primer can be immobilized to a support. Alternatively, an interior portion or the 3' end of a surface primer can be immobilized to a support.

[00298] The surface primers comprise DNA, RNA, or analogs thereof. The surface primers can include a combination of DNA and RNA. The sequence of surface primers can be wholly complementary or partially complementary along their length to at least a portion of the nucleic acid template molecule (e.g., linear or circular template molecules). A support can include a plurality of immobilized surface primers having the same sequence, or having two or more different sequences. Surface primers can be any length, for example 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths.

[00299] A surface primer can include a terminal 3' nucleotide having a sugar 3' OH moiety which is extendible for nucleotide polymerization (e.g., polymerase catalyzed polymerization). A surface primer can include a terminal 3' nucleotide having a moiety that blocks polymerase-catalyzed extension. A surface primer can include a terminal 3' nucleotide having the 3' sugar position linked to a chain-terminating moiety that inhibits nucleotide polymerization. The 3' chain-terminating moiety can be removed (e.g., de-blocked) to convert the 3' end to an extendible 3' OH end using a de-blocking agent. Examples of chain terminating moieties include alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group. Azide type chain terminating moieties including azide, azido and azidomethyl groups. Examples of de-blocking agents include a phosphine compound, such as Tris(2-carboxyethyl)phosphine (TCEP) and bis-sulfo triphenyl phosphine (BS-TPP), for chain-terminating groups azide, azido and azidomethyl groups. Examples of de-blocking agents include tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ), for chain-terminating groups alkyl, alkenyl, alkynyl and allyl. Examples of a de-blocking agent includes Pd/C for chain-terminating groups aryl and benzyl. Examples of de-blocking agents include

phosphine, beta-mercaptoethanol or dithiothritol (DTT), for chain-terminating groups amine, amide, keto, isocyanate, phosphate, thio and disulfide. Examples of de-blocking agents include potassium carbonate (K_2CO_3) in MeOH, triethylamine in pyridine, and Zn in acetic acid (AcOH), for carbonate chain-terminating groups. Examples of de-blocking agents include tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, and triethylamine trihydrofluoride, for chain-terminating groups urea and silyl.

[00300] In some embodiment, the plurality of immobilized surface capture primers on the support are in fluid communication with each other to permit flowing a solution of reagents (e.g., linear or circular nucleic acid template molecules, soluble primers, enzymes, nucleotides, divalent cations, buffers, reagents and the like) onto the support so that the plurality of immobilized surface capture primers on the support can be essentially simultaneously reacted with the reagents in a massively parallel manner. In some embodiments, the fluid communication of the plurality of immobilized surface capture primers can be used to conduct nucleic acid amplification reactions (e.g., RCA, MDA, PCR and bridge amplification) essentially simultaneously on the plurality of immobilized surface capture primers.

[00301] In some embodiment, the plurality of immobilized single stranded nucleic acid concatemer template molecules on the support are in fluid communication with each other to permit flowing a solution of reagents (e.g., soluble primers, enzymes, nucleotides, divalent cations, buffers, reagents and the like) onto the support so that the plurality of immobilized concatemer template molecules on the support can be essentially simultaneously reacted with the reagents in a massively parallel manner. In some embodiments, the fluid communication of the plurality of immobilized single stranded nucleic acid concatemer template molecules can be used to conduct nucleotide binding assays and/or conduct nucleotide polymerization reactions (e.g., primer extension or sequencing) essentially simultaneously on the plurality of immobilized single stranded nucleic acid concatemer template molecules, and optionally to conduct detection and imaging for massively parallel sequencing.

[00302] When used in reference to nucleic acids, the terms "amplify", "amplifying", "amplification", and other related terms include producing multiple copies of an original polynucleotide template molecule, where the copies comprise a sequence that is complementary to the template sequence, or the copies comprise a sequence that is the same as the template sequence. In some embodiments, the copies comprise a sequence that is substantially identical to

a template sequence, or is substantially identical to a sequence that is complementary to the template sequence.

[00303] The present disclosure provides various pH buffering agents. The full name of the pH buffering agents is listed herein.

The term "Tris" refers to a pH buffering agent Tris(hydroxymethyl)-aminomethane. The term "Tris-HCl" refers to a pH buffering agent Tris(hydroxymethyl)-aminomethane hydrochloride. The term "Tricine" refers to a pH buffering agent N-[tris(hydroxymethyl) methyl]glycine. The term "Bicine" refers to a pH buffering agent N,N-bis(2-hydroxyethyl)glycine. The term "Bis-Tris propane" refers to a pH buffering agent 1,3 Bis[tris(hydroxymethyl)methylamino]propane. The term "HEPES" refers to a pH buffering agent 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The term "MES" refers to a pH buffering agent 2-(*N*-morpholino)ethanesulfonic acid). The term "MOPS" refers to a pH buffering agent 3-(*N*-morpholino)propanesulfonic acid. The term "MOPSO" refers to a pH buffering agent 3-(N-morpholino)-2-hydroxypropanesulfonic acid. The term "BES" refers to a pH buffering agent N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid. The term "TES" refers to a pH buffering agent 2-[(2-Hydroxy-1,1bis(hydroxymethyl) ethyl)amino]ethanesulfonic acid). The term "CAPS" refers to a pH buffering agent 3-(cyclohexylamino)-1-propanesuhinic acid. The term "TAPS" refers to a pH buffering agent N-[Tris(hydroxymethyl)methyl]-3-amino propane sulfonic acid. The term "TAPSO" refers to a pH buffering agent N-[Tris(hydroxymethyl)methyl]-3-amino-2-hyidroxypropansulfonic acid. The term "ACES" refers to a pH buffering agent *N*-(2-Acetamido)-2-aminoethanesulfonic acid. The term "PIPES" refers to a pH buffering agent piperazine-1,4-bis(2-ethanesulfonic acid.

Introduction

[00304] The present disclosure provides compositions and methods that employ the compositions for conducting pairwise sequencing and for generating concatemer template molecules for pairwise sequencing.

[00305] Pairwise sequencing comprises obtaining a first sequencing read of a first region of a first nucleic acid strand (e.g., sense strand), and obtaining a second sequencing read of a second region of a second nucleic acid strand that is complementary to the first stand (e.g., anti-sense strand), wherein the first and second strands correspond to two complementary strands of the same double stranded template molecule. The first sequencing read of the first sequenced region

and the second sequencing read of the second sequenced region can having overlapping sequences which correspond to complementary sequences from the first and second strands of the double stranded template molecule. The first and second sequencing reads can be aligned so that the overlapping sequencing reads can yield sequence information of a paired region in the original double stranded nucleic acid source (e.g., a paired region in the genome), and the accuracy of the sequence information can be ascertained from the first and second sequencing reads with a high level of confidence. The first sequencing read of the first sequenced region and the second sequencing read of the second sequenced region do not necessarily have overlapping sequences in which case sequence information of a paired region in the original double stranded nucleic acid source cannot be ascertained with a high level of confidence. The first and second sequencing reads can initiate at one end of their respective template molecules, or can initiate at an internal position.

[00306] The compositions and methods for pairwise sequencing described herein offers several advantages which improves the quality of the sequencing data, including increased signal intensity which improves base call accuracy. The pairwise sequencing methods also saves time by obviating the need to prepare separate nucleic acid libraries each corresponding to the sense and anti-sense strands of the double stranded template molecule having the sequence of interest. Additionally, the pairwise sequencing methods generate and sequence the sense and anti-sense strands directly on the support/substrate used to conduct the sequencing reactions.

[00307] The present disclosure provides pairwise sequencing methods that employ a support having a plurality of surface primers immobilized thereon. The immobilized surface primers are in fluid communication with each other to permit flowing various solutions of linear or circular nucleic acid template molecules, soluble primers, enzymes, nucleotides, divalent cations, buffers, reagents, and the like, onto the support so that the plurality of immobilized surface primers (and products generated from the immobilized surface primers) react with the solutions in a massively parallel manner.

[00308] The present disclosure provides pairwise sequencing methods comprising the steps: **(a)** providing a plurality of single stranded nucleic acid concatemer template molecules immobilized to a support; **(b)** sequencing the plurality of immobilized concatemer template molecules with a first plurality of sequencing polymerases, a plurality of soluble forward sequencing primers and a first plurality of multivalent molecules, thereby generating a plurality

of extended forward sequencing primer strands; **(c)** retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized concatemer template molecules by conducting a primer extension reaction; **(d)** removing the retained immobilized concatemer template molecules while retaining the plurality of forward extension strands; and **(e)** sequencing the plurality of retained forward extension strands with a second plurality of sequencing polymerases, a plurality of soluble reverse sequencing primers and a second plurality of multivalent molecules. In some embodiments, individual concatemer template molecules in the plurality are immobilized to a surface primer where the surface primer is immobilized to the support. In some embodiments, individual concatemer template molecules are covalently joined to a surface primer, or individual concatemer template molecules are hybridized to a surface primer. In some embodiments, the immobilized surface primer includes or lacks a nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the surface primer. In some embodiments, the plurality of concatemer template molecules comprise at least one nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule. In some embodiments, the plurality of concatemer template molecules lack a nucleotide having a scissile moiety. Exemplary nucleotides having a scissile moiety (e.g., in the surface primer or the concatemer template molecule) include uridine, 8-oxo-7,8-dihydrogunine and deoxyinosine.

[00309] In some embodiments, pairwise sequencing methods include a rolling circle amplification reaction which is conducted on-support by distributing a plurality of single stranded circular library molecules onto the support having a plurality of surface primers immobilized thereon. Individual surface primers are designed to capture, via hybridization, a single circular library molecule. The rolling circle amplification reaction can be conducted on the support. In some embodiments, for the on-support RCA reaction, a solution of single stranded circular library molecules is flowed onto the support so that individual circular molecules are captured via hybridization to individual surface primers. Individual circular library molecules include at least a sequence of interest and a universal surface primer binding site, and optionally include universal sequencing primer binding sites, universal amplification primer binding site, an additional surface primer binding site, and a sample barcode and/or a molecular index. A single immobilized surface primer will capture a single circular library molecule and the rolling circle

amplification reaction generates a single stranded linear concatemer that is covalently linked to the immobilized surface primer by employing the terminal 3' end of the surface primer as a primer extension initiation site. Thus, individual concatemer molecules are immobilized to the support as concatemers that are covalently linked to an immobilized surface primer. The single stranded concatemer includes multiple tandem copies of the sequence of interest and the universal sequencing primer binding sites. A single surface primer will capture a single circular library molecule and generate a single concatemer molecule.

[00310] In some embodiments, pairwise sequencing methods include a rolling circle amplification reaction which is conducted in-solution to generate a plurality of concatemers which are distributed onto the support having a plurality of surface primers immobilized thereon. Individual surface primers are designed to capture, via hybridization, a single concatemer having complementary sequences of the circular library molecules. The rolling circle amplification reaction can continue on the support. In some embodiments, for the in-solution RCA reaction, a plurality of single stranded circular library molecules are subjected to a rolling circle amplification reaction in a reaction vessel. Individual circular library molecules include at least a sequence of interest and a universal surface primer binding site, and optionally include universal sequencing primer binding sites, universal amplification primer binding site, an additional surface primer binding site, and a sample barcode and/or a molecular index. The RCA reaction can be conducted for a very short period of time or can be conducted for longer periods of time, to generate a plurality of concatemers hybridized to their respective circular library molecules which are then distributed onto the support having a plurality of surface primers immobilized thereon. A solution of concatemer molecules is flowed onto the support so that individual concatemer molecules are captured via hybridization to individual surface primers. Individual concatemer molecules include at least a sequence of interest, universal surface primer binding site(s), universal sequencing primer binding sites, and optionally a sample barcode and/or a molecular index. A single immobilized surface primer will capture a single concatemer molecule and the rolling circle amplification reaction (now on the support) continues thereby extending the single stranded concatemer that is hybridized to the immobilized surface primer. Thus, individual concatemer molecules are immobilized to the support as concatemers that are hybridized to an immobilized surface primer. The single stranded concatemer includes multiple tandem copies of the sequence of interest and the universal sequencing primer binding sites. A single surface

primer will capture a single concatemer molecule and generate a single extended concatemer molecule.

[00311] The rolling circle amplification reaction, conducted either by in-solution or on-support, will generate concatemers that are immobilized to the support. Immobilized concatemers offer several advantages compared to non-concatemer molecules. The number of tandem copies in the concatemer is tunable by controlling the time, temperature and concentration of reagents of the in-solution or on-support rolling circle amplification reaction. The concatemer can self-collapse into a compact nucleic acid nanoball. Inclusion of one or more compaction oligonucleotides during the RCA reaction can further compact the size and/or shape of the nanoball. An increase in the number of tandem copies in a given concatemer increases the number of sites along the concatemer for hybridizing to multiple sequencing primers which serve as multiple initiation sites for polymerase-catalyzed sequencing reactions. When the sequencing reaction employs detectably labeled nucleotides and/or detectably labeled multivalent molecules (e.g., having nucleotide units), the signals emitted by the nucleotides or nucleotide units that participate in the parallel sequencing reactions along the concatemer yields an increased signal intensity for each concatemer. Multiple portions of a given concatemer can be simultaneously sequenced. Furthermore, a plurality of binding complexes can form along a particular concatemer molecule, each binding complex comprising a sequencing polymerase bound to a multivalent molecule wherein the plurality of binding complexes remain stable without dissociation resulting in increased persistence time which increases signal intensity and reduces imaging time.

[00312] The level of sequencing accuracy can be further improved by obtaining partially or wholly overlapping sequencing reads from both sense and anti-sense strands, and aligning the sequencing reads which provides redundant sequencing data.

[00313] Thus, the pairwise sequencing compositions and methods described herein provide improved sequencing data quality in a massively parallel manner.

Methods for Pairwise Sequencing – Generating Abasic Sites

[00314] The present disclosure provides pairwise sequencing methods, comprising **step (a):** providing a plurality of immobilized single stranded nucleic acid concatemer template molecules each comprising at least one nucleotide having a scissile moiety, wherein individual concatemer template molecules in the plurality are immobilized to a first surface primer that is immobilized

to a support, and wherein the immobilized first surface primer lacks a nucleotide having a scissile moiety. In some embodiments, the support comprises a plurality of first surface primers. In some embodiments, the support lacks a plurality of second surface primers. In some embodiments, the support comprises a plurality of first and second surface primers.

[00315] In some embodiments, individual immobilized concatemer template molecules are covalently joined to an immobilized surface primer (e.g., an immobilized first surface primer) (Figure 1). In an alternative embodiment, individual immobilized concatemer template molecules are hybridized to an immobilized surface primer (e.g., an immobilized first surface primer) (Figure 13).

[00316] In some embodiments, individual concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of: (i) two or more copies of a universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, (viii) two or more copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[00317] In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the forward sequencing primer can hybridize to at least a portion of the forward sequencing primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the reverse sequencing primer can hybridize to at least a portion of the reverse sequencing primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the immobilized first surface primer can hybridize to at least a portion of the immobilized first surface primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the immobilized second surface primer can hybridize to at least a portion of the immobilized second surface primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the first

soluble amplification primer can hybridize to at least a portion of the first soluble amplification primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the second soluble amplification primer can hybridize to at least a portion of the second soluble amplification primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the soluble compaction oligonucleotide can hybridize to at least a portion of the soluble compaction oligonucleotide.

[00318] In some embodiments, the scissile moiety in the immobilized concatemer template molecules of step (a) can be converted into abasic sites in the immobilized concatemer template molecules. In some embodiments, the scissile moiety in the immobilized concatemer template molecules comprises uridine, 8-oxo-7,8-dihydroguanine (e.g., 8oxoG) or deoxyinosine. In the concatemer template molecules, the uridine can be converted to an abasic site using uracil DNA glycosylase (UDG), the 8oxoG can be converted to an abasic site using FPG glycosylase, and the deoxyinosine can be converted to an abasic site using AlkA glycosylase. In some embodiments, the immobilized concatemer template molecules include 1-20, 20-40, 40-60, 60-80, 80-100, or a higher number of nucleotides with a scissile moiety. In some embodiments, about 0.1-1%, or about 1-5%, or about 5-10%, or about 10-20%, or about 20-30% or a higher percent of the dTTP in the immobilized concatemer template molecules are replaced with nucleotides having a scissile moiety. In some embodiments, the nucleotides having a scissile moiety are distributed at random positions along individual immobilized concatemer template molecules. In some embodiments, the nucleotides having a scissile moiety are distributed at different positions in the different immobilized concatemer template molecules.

[00319] In some embodiments, the immobilized first surface primers comprise single stranded oligonucleotides comprising DNA, RNA or a combination of DNA and RNA. The immobilized first surface primers can be immobilized to the support or immobilized to a coating on the support. The immobilized first surface primers can be embedded and attached (coupled) to the coating on the support. In some embodiments, the 5' end of the immobilized first surface primers are immobilized to a support or immobilized to a coating on the support. Alternatively, an interior portion or the 3' end of the immobilized first surface primers can be immobilized to a support or immobilized to a coating on the support. The support comprises a plurality of immobilized first surface primers having the same sequence. The immobilized first surface primers can be any length, for example 4-50 nucleotides, or 50-100 nucleotides, or 100-150

nucleotides, or longer lengths. In some embodiments, the 3' terminal end of the immobilized first surface primers comprise an extendible 3' OH moiety. In some embodiments, the 3' terminal end of the immobilized first surface primers comprise a 3' non-extendible moiety.

[00320] In some embodiments, the plurality of immobilized first surface primers comprise at least one phosphorothioate diester bond at their 5' ends which can render the first surface primers resistant to exonuclease degradation. In some embodiments, the plurality of immobilized first surface primers comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5' ends. In some embodiments, the plurality of immobilized first surface primers comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the first surface primers resistant to exonuclease degradation.

[00321] In some embodiments, the immobilized first surface primers comprise at least one locked nucleic acid (LNA) which comprises a methylene bridge bond between a 2' oxygen and 4' carbon of the pentose ring. Immobilized first surface primers that include at least one LNA can be resistant to nuclease digestions and can exhibit increased melting temperature when hybridized to the forward extension strand.

[00322] In some embodiments, the immobilized concatemer template molecules further comprise two or more copies of a universal binding sequence (or complementary sequence thereof) for an immobilized second surface primer having a sequence that differs from the first immobilized surface primer. The immobilized second surface primers of step (a) comprise single stranded oligonucleotides comprising DNA, RNA or a combination of DNA and RNA. The immobilized second surface primers can be immobilized to the support or immobilized to a coating on the support. The immobilized second surface primers can be embedded and attached (coupled) to the coating on the support. In some embodiments, the 5' end of the immobilized second surface primers are immobilized to a support or immobilized to a coating on the support. Alternatively, an interior portion or the 3' end of the immobilized second surface primers can be immobilized to a support or immobilized to a coating on the support. The support comprises a plurality of immobilized second surface primers having the same sequence. The immobilized second surface primers can be any length, for example 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths.

[00323] In some embodiments, the 3' terminal end of the immobilized second surface primers comprise an extendible 3' OH moiety. In some embodiments, the 3' terminal end of the

immobilized second surface primers comprise a 3' non-extendible moiety. In some embodiments, the 3' terminal end of the immobilized second surface primers comprise a moiety that blocks primer extension (e.g., non-extendible terminal 3' end), such as for example a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group. The immobilized second surface primers are not extendible in a primer extension reaction. The immobilized second surface primers lack a nucleotide having a scissile moiety.

[00324] In some embodiments, the plurality of immobilized second surface primers comprise at least one phosphorothioate diester bond at their 5' ends which can render the second surface primers resistant to exonuclease degradation. In some embodiments, the plurality of immobilized second surface primers comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5' ends. In some embodiments, the plurality of immobilized second surface primers comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the second surface primers resistant to exonuclease degradation.

[00325] In some embodiments, individual immobilized single stranded nucleic acid concatemer template molecule are joined or immobilized to an immobilized first surface primer, and at least one portion of the individual concatemer template molecule is hybridized to an immobilized second surface primer. The immobilized second surface primers serve to pin down a portion of the immobilized concatemer template molecules to the support (see Figures 12 and 24).

[00326] In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized first surface primers per mm^2. In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized second surface primers per mm^2. In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized first surface primers and immobilized second surface primers per mm^2.

[00327] The immobilized surface primers (e.g., first and second surface primers) are in fluid communication with each other to permit flowing various solutions of linear or circular nucleic acid template molecules, soluble primers, enzymes, nucleotides, divalent cations, buffers, reagents, and the like, onto the support so that the plurality of immobilized surface primers (and the primer extension products generated from the immobilized surface primers) react with the solutions in a massively parallel manner.

[00328] In some embodiments, the pairwise sequencing method further comprises **step (b):** sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands. The sequencing of step (b) comprises contacting the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers under a condition suitable to hybridize at least one forward sequencing primer to at least one of the forward sequencing primer binding sites/sequences of the immobilized concatemer template molecules, and conducting forward sequencing reactions using one or more types of sequencing polymerases, a plurality of nucleotides and/or multivalent molecules, and the hybridized first forward sequencing primers. The forward sequencing reactions can generate a plurality of extended forward sequencing primer strands. In some embodiments, individual immobilized concatemer template molecules have multiple copies of the forward sequencing primer binding sites, wherein each forward sequencing primer binding site is capable of hybridizing to a first forward sequencing primer. Individual forward sequencing primer binding sites in a given immobilized concatemer template molecule can be hybridized to a forward sequencing primer and can undergo a sequencing reaction. Individual immobilized concatemer template molecules can undergo two or more sequence reactions, where each sequencing reaction is initiated from a first forward sequencing primer that is hybridized to a forward sequencing primer binding site (e.g., see Figures 2 and 14). In some embodiments, the soluble forward sequencing primers comprise 3' OH extendible ends. In some embodiments, the soluble forward sequencing primers comprise a 3' blocking moiety which can be removed to generate a 3' OH extendible end. In some embodiments, the soluble forward sequencing primers lack a nucleotide having a scissile moiety. In some embodiments, the sequencing reactions comprise a plurality of nucleotides (or analogs thereof) labeled with a detectable reporter moiety. In some embodiments, the sequencing reaction comprise a plurality of multivalent molecules having a plurality of nucleotide units attached to a core, where the multivalent molecules are labeled with a detectable reporter moiety. In some embodiments, the core is labeled with a detectable reporter moiety. In some embodiments, at least one linker and/or at least one nucleotide unit of a nucleotide arm is labeled with a detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore. An exemplary nucleotide arm is shown in Figure 108, and exemplary multivalent molecules are shown in Figures 104-107.

[00329] In some embodiments, the pairwise sequencing method further comprises **step (c):** retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules. The plurality of extended forward sequencing primer strands can be removed and replaced with a plurality of forward extension strands by conducting a primer extension reaction (see Figures 3-5, and Figures 15-17).

[00330] In some embodiments, step (c) comprises contacting at least one extended forward sequencing primer strand with a plurality of strand displacing polymerases and a plurality of nucleotides and in the absence of soluble amplification primers, under a condition suitable to conduct a strand displacing primer extension reaction using the at least one extended forward sequencing primers strand to initiate the primer extension reaction thereby generating a forward extension strand that is covalently joined to the extended forward sequencing primers strand, wherein the forward extension strand is hybridized to the immobilized concatemer template molecule. For example, one of the extended forward sequencing primer strands can serve as a primer for the strand displacing polymerase. The strand displacing polymerase can extend the extended forward sequencing primer strand, and displace downstream extended forward sequencing primer strands while synthesizing an extended strand that replaces the downstream extended forward sequencing primer strands (Figures 3 and 15). The newly extended strand is covalently joined to an extended forward sequencing primer strand. The immobilized concatemer template molecules are retained.

[00331] The primer extension reaction can optionally include a plurality of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) to generate forward extension strands. Individual forward extension strands can collapse into a nanoball having a more compact size and/or shape compared to a nanoball generated from a primer extension reaction conducted without compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III). Inclusion of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) in the primer extension reaction can improve FWHM (full width half maximum) of a spot image of the nanoball. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

[00332] Examples of strand displacing polymerases include phi29 DNA polymerase, large fragment of Bst DNA polymerase, large fragment of Bsu DNA polymerase (exo-), Bca DNA polymerase (exo-), Klenow fragment of E. coli DNA polymerase, T5 polymerase, M-MuLV reverse transcriptase, HIV viral reverse transcriptase, Deep Vent DNA polymerase and KOD DNA polymerase. The phi29 DNA polymerase can be wild type phi29 DNA polymerase (e.g., MagniPhi from Expedeon), or variant EquiPhi29 DNA polymerase (e.g., from Thermo Fisher Scientific), or chimeric QualiPhi DNA polymerase (e.g., from 4basebio).

[00333] In some embodiments, step (c) comprises: **(i)** removing the plurality of extended forward sequencing primer strand while retaining the immobilized concatemer template molecules; and **(ii)** contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble forward sequencing primers (e.g., a second plurality of soluble forward sequencing primers), a plurality of nucleotides (e.g., a second plurality of nucleotides) and a plurality of primer extension polymerases, under a condition suitable to hybridize the plurality of soluble forward sequencing primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed primer extension reactions thereby generating a plurality of forward extension strands, wherein the soluble sequencing primers hybridize with the forward sequencing primer binding sequence in the retained immobilized concatemer molecules (Figures 4 and 16). The primer extension reaction can optionally include a plurality of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) to generate forward extension strands. Individual forward extension strands can collapse into a nanoball having a more compact size and/or shape compared to a nanoball generated from a primer extension reaction conducted without compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III). Inclusion of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) in the primer extension reaction can improve FWHM (full width half maximum) of a spot image of the nanoball. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

[00334] In some embodiments, in step (c), the condition suitable to hybridize the plurality of soluble forward sequencing primers to the plurality of retained immobilized single stranded nucleic acid concatemer template molecules comprises hybridizing retained immobilized

concatemer template molecules with the soluble primers in the presence of a primer extension polymerase, a plurality of nucleotides, and a high efficiency hybridization buffer. In some embodiment, the high efficiency hybridization buffer comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

[00335] In some embodiments, step (c) comprises: **(i)** removing the plurality of extended forward sequencing primer strand while retaining the immobilized concatemer template molecules; and **(ii)** contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble amplification primers, a plurality of nucleotides (e.g., a second plurality of nucleotides) and a plurality of primer extension polymerases, under a condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed primer extension reactions thereby generating a plurality of forward extension strands, wherein the soluble amplification primers hybridize with the soluble amplification primer binding sequence in the retained immobilized concatemer molecules (Figures 5 and 17). The primer extension reaction can optionally include a plurality of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) to generate forward extension strands. Individual forward extension strands can collapse into a nanoball having a more compact size and/or shape compared to a nanoball generated from a primer extension reaction conducted without compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III). Inclusion of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) in the primer extension reaction can improve FWHM (full

width half maximum) of a spot image of the nanoball. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

[00336] In some embodiments, in step (c), the condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized single stranded nucleic acid concatemer template molecules comprises hybridizing retained immobilized concatemer template molecules with the soluble primers in the presence of a primer extension polymerase, a plurality of nucleotides, and a high efficiency hybridization buffer. In some embodiment, the high efficiency hybridization buffer comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

[00337] In some embodiments, in step (c), the plurality of extended forward sequencing primer strands can be removed using an enzyme or a chemical reagent. For example, the plurality of extended forward sequencing primer strands can be enzymatically degraded using a 5' to 3' double-stranded DNA exonuclease, including T7 exonuclease (e.g., from New England Biolabs, catalog # M0263S). In some embodiments, the plurality of extended forward sequencing primer strands can be removed with a temperature that favors nucleic acid denaturation.

[00338] In some embodiments, in step (c), a denaturation reagent can be used to remove the plurality of extended forward sequencing primer strands, wherein the denaturation reagent

comprises any one or any combination of compounds such as formamide, acetonitrile, guanidinium chloride and/or a pH buffering agent (e.g., Tris-HCl, MES, HEPES, MOPS, or the like). Optionally, the denaturation reagent can further comprise PEG.

[00339] In some embodiments, in step (c), the plurality of extended forward sequencing primer strands can be removed using an elevated temperature (e.g., heat) with or without a nucleic acid denaturation reagent. The plurality of extended forward sequencing primer strands can be subjected to a temperature of about 45-50 °C, or about 50-60 °C, or about 60-70 °C, or about 70-80 °C, or about 80-90 °C, or about 90-95 °C, or higher temperature.

[00340] In some embodiments, in step (c), the plurality of extended forward sequencing primer strands can be removed using 100% formamide at a temperature of about 65 °C for about 3 minutes, and washing with a reagent comprising about 50 mM NaCl or equivalent ionic strength and having a pH of about 6.5 – 8.5.

[00341] In some embodiments, the primer extension polymerase of step (c) comprises a high fidelity polymerase. In some embodiments, the primer extension polymerase of step (c) comprises a DNA polymerase capable of catalyzing a primer extension reaction using a uracil-containing template molecule (e.g., a uracil-tolerant polymerase). Exemplary polymerases include, but are not limited to, Q5U Hot Start high-fidelity DNA polymerase (e.g., catalog # M0515S from New England Biolabs), Taq DNA polymerase, One Taq DNA polymerase (e.g., mixture of Taq and Deep Vent DNA polymerases, catalog #M0480S from New England Biolabs), LongAmp Taq DNA polymerase (e.g., catalog #M0323S from New England Biolabs), Epimark Hot Start Taq DNA polymerase (e.g., catalog #M0490S from New England Biolabs), Bst DNA polymerase (e.g., large fragment, catalog #M0275S from New England Biolabs), Bsu DNA polymerase (e.g., large fragment, catalog #M0330S from New England Biolabs), Phi29 DNA polymerase (e.g., catalog # M0269S from New England Biolabs), *E. coli* DNA polymerase (e.g., catalog # M0209S from New England Biolabs), Therminator DNA polymerase (e.g., catalog #M0261S from New England Biolabs), Vent DNA polymerase and Deep Vent DNA polymerase.

[00342] The pairwise methods described herein can provide increased accuracy in a downstream sequencing reaction because step (c) replaces the extended forward sequencing primer strands that were generated in step (b) with forward extension strands having reduced base errors. The extended forward sequencing primer strands are generated in step (b) and may

or may not contain erroneously incorporated nucleotides due to polymerase-catalyzed mis-paired bases. When step (c) is conducted with a high fidelity DNA polymerase, the resulting forward extension strands may have reduced base errors compared to the extended forward sequencing primer strands. The forward extension strands will be used as a nucleic acid template for a downstream sequencing step (e.g., see step (e) below). Thus, step (c) can increase the sequencing accuracy of the downstream step (e) and therefore increase the overall sequencing accuracy of the pairwise sequencing workflow.

[00343] In some embodiments, the pairwise sequencing method further comprises **step (d):** removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized surface primers (Figures 6 and 18).

[00344] The abasic sites are generated on the retained concatemer template strands that contain nucleotides having scissile moieties. In some embodiments, the scissile moieties in the retained concatemer template molecules comprises uridine, 8-oxo-7,8-dihydroguanine (e.g., 8oxoG) or deoxyinosine. The abasic sites can be removed to generate a plurality of single stranded nucleic acid template molecules having gaps while retaining the plurality of forward extension strands. The abasic sites can be generated by contacting the immobilized concatemer template molecules with an enzyme that removes the nucleo-base at the nucleotide having the scissile moiety. The uracil in the retained concatemer template strands can be converted to an abasic site using uracil DNA glycosylase (UDG). The 8oxoG in the retained concatemer template strands can be converted to an abasic site using FPG glycosylase. The deoxyinosine in the retained concatemer template strands can be converted to an abasic site using AlkA glycosylase.

[00345] In some embodiments, in step (d), the gaps can be generated by contacting the abasic sites in the immobilized concatemer template molecules with an enzyme or a mixture of enzymes having lyase activity that breaks the phosphodiester backbone at the 5' and 3' sides of the abasic site to release the base-free deoxyribose and generate a gap (Figures 6 and 18). The abasic sites can be removed using AP lyase, Endo IV endonuclease, FPG glycosylase/AP lyase, Endo VIII

glycosylase/AP lyase. In some embodiments, generating the abasic sites and removal of the abasic sites to generate gaps can be achieved using a mixture of uracil DNA glycosylase and DNA glycosylase-lyase endonuclease VIII, for example USER (Uracil-Specific Excision Reagent Enzyme from New England Biolabs) or thermolabile USER (also from New England Biolabs).

[00346] In some embodiments, in step (d), the plurality of gap-containing template molecules can be removed using an enzyme, chemical and/or heat. After the gap-removal procedure, the plurality of retained forward extension strands (e.g., see Figures 7 and 9, and Figures 19 and 21). is hybridized to the retained immobilized surface primers

[00347] For example, the plurality of gap-containing template molecules can be enzymatically degraded using a 5' to 3' double-stranded DNA exonuclease, including T7 exonuclease (e.g., from New England Biolabs, catalog # M0263S). When a 5' to 3' double-stranded DNA exonuclease is used for removing gap-containing template molecules, then the plurality of soluble amplification primers in step (c) can comprise at least one phosphorothioate diester bond at their 5' ends which can render the soluble amplification primers resistant to exonuclease degradation. In some embodiments, the plurality of soluble amplification primers in step (c) comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5' ends. In some embodiments, the plurality soluble amplification primers in step (c) comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the forward sequencing primers resistant to exonuclease degradation.

[00348] In some embodiments, the plurality of gap-containing template molecules can be removed using a chemical reagent that favors nucleic acid denaturation. The denaturation reagent can include any one or any combination of compounds such as formamide, acetonitrile, guanidinium chloride and/or a buffering agent (e.g., Tris-HCl, MES, HEPES, or the like).

[00349] In some embodiments, the plurality of gap-containing template molecules can be removed using an elevated temperature (e.g., heat) with or without a nucleic acid denaturation reagent. The gap-containing template molecules can be subjected to a temperature of about 45-50 °C, or about 50-60 °C, or about 60-70 °C, or about 70-80 °C, or about 80-90 °C, or about 90-95 °C, or higher temperature.

[00350] In some embodiments, the plurality of gap-containing template molecules can be removed using 100% formamide at a temperature of about 65 °C for about 3 minutes, and

washing with a reagent comprising about 50 mM NaCl or equivalent ionic strength and having a pH of about 6.5 – 8.5.

[00351] In some embodiments, the pairwise sequencing method further comprises **step (e):** sequencing the plurality of retained forward extension strands thereby generating a plurality of extended reverse sequencing primer strands. In some embodiments, the sequencing of step (e) comprises contacting the plurality of retained forward extension strands with a plurality of soluble reverse sequencing primers under a condition suitable to hybridize the reverse sequencing primers to the reverse sequencing primer binding site of the retained forward extension strands, and by conducting sequencing reactions using the hybridized reverse sequencing primers wherein the forward sequencing reactions generates a plurality of extended reverse sequencing primer strands (Figures 10 and 11, and Figures 22 and 23). The extended reverse sequencing primer strands are hybridized to the retained forward extension strand. The retained forward extension strand is hybridized to the first surface primer. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support.

[00352] For the sake of simplicity, Figures 7 and 9 show exemplary retained forward extension strands each having one copy of the sequence of interest and various universal primer binding sites. The skilled artisan will appreciate that the retained forward extension strand can include two or more tandem copies containing the sequence of interest and various universal primer binding sites. Therefore, the reverse sequencing reaction can generate a plurality of extended reverse sequencing primer strands hybridized to the same retained forward extension strand.

[00353] In some embodiments, in step (e), the condition suitable to hybridize the reverse sequencing primers to the reverse sequencing primer binding sequences of the retained forward extension strands comprises contacting the plurality of soluble reverse sequencing primers and the retained forward extension strands with a high efficiency hybridization buffer. In some embodiments, the high efficiency hybridization buffer comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded

nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

[00354] In an alternative embodiment, the sequencing of step (e) comprises using the immobilized surface primer as a sequencing primer and conducting sequencing reactions to generate a plurality of reverse sequencing strands.

[00355] In some embodiments, the reverse sequencing reactions of step (e) comprises contacting the plurality of soluble reverse sequencing primers with the reverse sequencing primer binding sequences of the retained forward extension strands, one or more types of sequencing polymerases, and a plurality of nucleotides or a plurality of multivalent molecules. In some embodiments, the soluble reverse sequencing primers comprise 3' OH extendible ends. In some embodiments, the soluble reverse sequencing primers comprise a 3' blocking moiety which can be removed to generate a 3' OH extendible end. In some embodiments, the soluble reverse sequencing primers lack a nucleotide having a scissile moiety. The sequencing reactions that employ nucleotides and/or multivalent molecules is described in more detail below. The reverse sequencing reactions can generate a plurality of extended reverse sequencing primer strands. In some embodiments, individual retained forward extension strands have multiple copies of the reverse sequencing primer binding sequences/sites, wherein each reverse sequencing primer binding site is capable of hybridizing to a reverse sequencing primer. Individual reverse sequencing primer binding sites in a given retained forward extension strand can be hybridized to a reverse sequencing primer and can undergo a sequencing reaction. Thus, an individual retained forward extension strand can undergo two or more sequence reactions, where each sequencing reaction is initiated from a reverse sequencing primer that is hybridized to a reverse sequencing primer binding site (e.g., see Figures 10 and 11, and Figures 22 and 23). In some embodiments, the sequencing reactions comprise a plurality of nucleotides (or analogs thereof) labeled with a

detectable reporter moiety. In some embodiments, the sequencing reaction comprise a plurality of multivalent molecules having nucleotide units, where the multivalent molecules are labeled with a detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore.

[00356] In some embodiments, at least one washing step can be conducted after any of steps (a) – (e). The washing step can be conducted with a wash buffer comprising a pH buffering agent, a metal chelating agent, a salt, and a detergent.

[00357] In some embodiments, the pH buffering compound in the wash buffer comprises any one or any combination of two or more of Tris, Tris-HCl, Tricine, Bicine, Bis-Tris propane, HEPES, MES, MOPS, MOPSO, BES, TES, CAPS, TAPS, TAPSO, ACES, PIPES, ethanolamine (a.k.a 2-amino methanol; MEA), a citrate compound, a citrate mixture, NaOH and/or KOH. In some embodiments, the pH buffering agent can be present in the wash buffer at a concentration of about 1-100 mM, or about 10-50 mM, or about 10-25 mM. In some embodiments, the pH of the pH buffering agent which is present in any of the reagents described here in can be adjusted to a pH of about 4-9, or a pH of about 5-9, or a pH of about 5-8.

[00358] In some embodiments, the metal chelating agent in the wash buffer comprises EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol tetraacetic acid), HEDTA (hydroxyethylethylenediaminetriacetic acid), DPTA (diethylene triamine pentaacetic acid), NTA (N,N-bis(carboxymethyl)glycine), citrate anhydrous, sodium citrate, calcium citrate, ammonium citrate, ammonium bicitrate, citric acid, potassium citrate, or magnesium citrate. In some embodiments, the wash buffer comprises a chelating agent at a concentration of about 0.01 – 50 mM, or about 0.1 – 20 mM, or about 0.2 – 10 mM.

[00359] In some embodiments, the salt in the wash buffer comprises NaCl, KCl, NH_2SO_4 or potassium glutamate. In some embodiments, the detergent comprises an ionic detergent such as SDS (sodium dodecyl sulfate). The wash buffer can include a monovalent salt at a concentration of about 25-500 mM, or about 50-250 mM, or about 100-200 mM.

[00360] In some embodiments, the detergent in the wash buffer comprises a non-ionic detergent such as Triton X-100, Tween 20, Tween 80 or Nonidet P-40. In some embodiments, the detergent comprises a zwitterionic detergent such as CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) or *N*-Dodecyl-*N,N*-dimethyl-3-amonio-1-propanesulfate (DetX). In some embodiments, the detergent comprises LDS (lithium

dodecyl sulfate), sodium taurodeoxycholate, sodium taurocholate, sodium glycocholate, sodium deoxycholate or sodium cholate. In some embodiments, the detergent is included in the wash buffer at a concentration of about 0.01-0.05%, or about 0.05-0.1%, or about 0.1-0.15%, or about 0.15-0.2%, or about 0.2-0.25%.

On Support RCA and Pairwise Sequencing – Generating Abasic Sites

[00361] The present disclosure provides pairwise sequencing methods, comprising **step (a):** providing a support having a plurality of surface primers (e.g., a plurality of first surface primers) immobilized thereon wherein each of the surface primers have a 3' OH extendible end and lack a nucleotide having a scissile moiety (Figure 25). For example, the surface primers lack uridine, 8-oxo-7,8-dihydroguanine (e.g., 8oxoG) and deoxyinosine. In some embodiments, the support comprises a plurality of first surface primers. In some embodiments, the support lacks a plurality of second surface primers. In some embodiments, the support comprises a plurality of first and second surface primers.

[00362] In some embodiments, the immobilized first surface primers comprise single stranded oligonucleotides comprising DNA, RNA or a combination of DNA and RNA. The first surface primers comprise a sequence that is wholly complementary or partially complementary along their lengths to at least a portion of a nucleic acid library molecule (e.g., linear or circular library molecules). The first surface primers can include a terminal 3' nucleotide having a sugar 3' OH moiety which is extendible for nucleotide polymerization (e.g., polymerase catalyzed polymerization).

[00363] The immobilized first surface primers can be immobilized to the support or immobilized to a coating on the support. The immobilized first surface primers can be embedded and attached (coupled) to the coating on the support. In some embodiments, the 5' end of the immobilized first surface primers are immobilized to a support or immobilized to a coating on the support. Alternatively, an interior portion or the 3' end of the immobilized first surface primers can be immobilized to a support or immobilized to a coating on the support. The support comprises a plurality of immobilized first surface primers having the same sequence. The immobilized first surface primers can be any length, for example 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths.

[00364] In some embodiments, the plurality of immobilized first surface primers comprise at least one phosphorothioate diester bond at their 5' ends which can render the first surface

primers resistant to exonuclease degradation. In some embodiments, the plurality of immobilized first surface primers comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5' ends. In some embodiments, the plurality of immobilized first surface primers comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the first surface primers resistant to exonuclease degradation.

[00365] In some embodiments, the immobilized first surface primers comprise at least one locked nucleic acid (LNA) which comprises a methylene bridge bond between a 2' oxygen and 4' carbon of the pentose ring. Immobilized first surface primers that include at least one LNA can be resistant to nuclease digestions and can exhibit increased melting temperature when hybridized to the forward extension strand.

[00366] In some embodiments, the support further comprises a plurality of a second surface primer immobilized thereon (Figure 37). The second surface primers have a sequence that differs from the first immobilized surface primer. The immobilized second surface primers of step (a) comprise single stranded oligonucleotides comprising DNA, RNA or a combination of DNA and RNA. The second surface primers comprise a sequence that is wholly complementary or partially complementary along their lengths to at least a portion of an immobilized single stranded concatemer template molecule. The immobilized second surface primers can be immobilized to the support or immobilized to a coating on the support. The immobilized second surface primers can be embedded and attached (coupled) to the coating on the support. In some embodiments, the 5' end of the immobilized second surface primers are immobilized to a support or immobilized to a coating on the support. Alternatively, an interior portion or the 3' end of the immobilized second surface primers can be immobilized to a support or immobilized to a coating on the support. The support comprises a plurality of immobilized second surface primers having the same sequence. The immobilized second surface primers can be any length, for example 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths. In some embodiments, the 3' terminal end of the immobilized second surface primers comprise an extendible 3' OH moiety. In some embodiments, the 3' terminal end of the immobilized second surface primers comprise a 3' non-extendible moiety. The 3' terminal end of the immobilized second surface primers comprise a moiety that blocks primer extension, such as for example a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group. The immobilized

second surface primers are not extendible in a primer extension reaction. The immobilized second surface primers lack a nucleotide having a scissile moiety.

[00367] In some embodiments, the plurality of immobilized second surface primers comprise at least one phosphorothioate diester bond at their 5' ends which can render the second surface primers resistant to exonuclease degradation. In some embodiments, the plurality of immobilized second surface primers comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5' ends. In some embodiments, the plurality of immobilized second surface primers comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the second surface primers resistant to exonuclease degradation.

[00368] In some embodiments, individual immobilized single stranded nucleic acid concatemer template molecule are covalently joined to an immobilized first surface primer, and at least one portion of the individual concatemer template molecule is hybridized to an immobilized second surface primer (Figure 37). The immobilized second surface primers serve to pin down a portion of the immobilized concatemer template molecules to the support. The immobilized concatemer template molecule has two or more copies of a universal binding sequence for an immobilized second surface primer. The portion of the immobilized concatemer template molecule that includes the universal binding sequence for an immobilized second surface primer can hybridize to the immobilized second surface primer. In some embodiments, the second surface primers include a terminal 3' blocking group that renders them non-extendible. In some embodiments, the second surface primers have terminal 3' extendible ends.

[00369] In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized first surface primers per mm^2. In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized second surface primers per mm^2. In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized first surface primers and immobilized second surface primers per mm^2.

[00370] The immobilized surface primers (e.g., first and second surface primers) are in fluid communication with each other to permit flowing various solutions of linear or circular nucleic acid template molecules, soluble primers, enzymes, nucleotides, divalent cations, buffers, reagents, and the like, onto the support so that the plurality of immobilized surface primers (and the primer extension products generated from the immobilized surface primers) react with the solutions in a massively parallel manner.

[00371] In some embodiments, the pairwise sequencing method further comprises **step (b):** generating a plurality of immobilized single stranded nucleic acid concatemer template molecules wherein individual single stranded nucleic acid concatemer template molecules are joined (e.g., covalently joined) to an immobilized surface primer (e.g., an immobilized first surface primer), by hybridizing a plurality of single-stranded circular nucleic acid library molecules to the plurality of immobilized first surface primers and conducting a rolling circle amplification reaction with a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety, thereby generating a plurality of immobilized single stranded nucleic acid concatemer template molecules (Figure 26). In some embodiments, the rolling circle amplification reaction can be conducted in the presence, or in the absence, of a plurality of compaction oligonucleotides.

[00372] In some embodiments, the single-stranded circular nucleic acid library molecules comprise covalently closed circular molecules. In some embodiments, the single-stranded circular nucleic acid library molecules can be removed from the concatemer template molecules with at least one washing step which is conducted under a condition suitable to retain the single stranded nucleic acid concatemer template molecules where individual concatemer template molecules are operably joined to an immobilized first surface primer.

[00373] In some embodiments, each of the single stranded circular nucleic acid library molecules in the plurality comprise a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of (i) a universal binding sequence (or complementary sequence thereof) for a soluble forward sequencing primer, (ii) a universal binding sequence (or complementary sequence thereof) for a soluble reverse sequencing primer, (iii) a universal binding sequence (or complementary sequence thereof) for an immobilized first surface primer, (iv) a universal binding sequence (or complementary sequence thereof) for an immobilized second surface primer, (v) a universal binding sequence (or complementary sequence thereof) for a first soluble amplification primer, (vi) a universal binding sequence (or complementary sequence thereof) for a second soluble amplification primer, (vii) a universal binding sequence (or complementary sequence thereof) for a soluble compaction oligonucleotide, (viii) a sample barcode sequence and/or (ix) a unique molecular index sequence.

[00374] In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the forward sequencing primer can hybridize to at least a portion of the forward sequencing primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the reverse sequencing primer can hybridize to at least a portion of the reverse sequencing primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the immobilized first surface primer can hybridize to at least a portion of the immobilized first surface primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the immobilized second surface primer can hybridize to at least a portion of the immobilized second surface primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the first soluble amplification primer can hybridize to at least a portion of the first soluble amplification primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the second soluble amplification primer can hybridize to at least a portion of the second soluble amplification primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the soluble compaction oligonucleotide can hybridize to at least a portion of the soluble compaction oligonucleotide.

[00375] In some embodiments, the rolling circle amplification reaction of step (b) generates a plurality of immobilized single stranded nucleic acid concatemer template molecules each comprising a concatemer having at least one nucleotide having a scissile moiety and two or more copies of a sequence of interest, and wherein the immobilized concatemer template molecules further comprise any one or any combination of two or more of: (i) two or more copies of a universal binding sequence (or a complementary sequence thereof) for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence (or a complementary sequence thereof) for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence (or a complementary sequence thereof) for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence (or a complementary sequence thereof) for an immobilized second surface primer, (v) two or more copies of a universal binding sequence (or a complementary sequence thereof) for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence (or a complementary sequence thereof) for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence (or a complementary sequence thereof) for a soluble compaction oligonucleotide, (viii) two or more

copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[00376] In some embodiments, the plurality of immobilized single stranded nucleic acid concatemer template molecules that are generated by the rolling circle amplification reaction of step (b) further comprise two or more copies of a universal binding sequence (or complementary sequence thereof) for immobilized second sequence surface primers. In some embodiments, individual immobilized single stranded nucleic acid concatemer template molecule are joined (e.g., covalently joined) to an immobilized first surface primer, and at least one portion of the individual concatemer template molecule is hybridized to an immobilized second surface primer. The immobilized second surface primers serve to pin down a portion of the immobilized concatemer template molecules to the support (see Figure 37). In some embodiments, the second surface primers include a terminal 3' blocking group that renders them non-extendible.

[00377] The rolling circle amplification reaction of step (b) can be conducted with a nucleotide mixture containing dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety to generate immobilized concatemer template molecules which includes at least one nucleotide having a scissile moiety. The scissile moieties in the immobilized concatemer template molecules can be converted into abasic sites. In some embodiments, in the nucleotide mixture, the nucleotide having the scissile moiety comprises uridine, 8-oxo-7,8-dihydroguanine (e.g., 8oxoG) or deoxyinosine. In the immobilized concatemer template molecules, the uridine can be converted to an abasic site using uracil DNA glycosylase (UDG), the 8oxoG can be converted to an abasic site using FPG glycosylase, and the deoxyinosine can be converted to an abasic site using AlkA glycosylase.

[00378] In some embodiments, the nucleotide mixture can include an amount of dUTP so that a target percent of the thymidine in the resulting concatemer molecules are replaced with dUTP. For example, when 30% of dTTP in the concatemer molecules are to be replaced with dUTP (e.g., 30% is the target percent) then the nucleotide mixture can contain 7.5% dUTP (e.g., 30/4 = 7.5%), 17.5% dTTP, and 25% each for dATP, dCTP and dGTP. The target percent of dTTP to be replaced by dUTP can be about 0.1-1%, or about 1-5%, or about 5-10%, or about 10-20%, or about 20-30% , or about 30-45%, or about 45-50%, or a higher percent of the dTTP in the immobilized concatemer template molecules are replaced with nucleotides having a scissile moiety.

[00379] In some embodiments, the nucleotide mixture can include an amount of deoxyinosine so that a target percent of the guanosine in the resulting concatemer molecules are replaced with deoxyinosine. For example, when 30% of dGTP in the concatemer molecules are to be replaced with deoxyinosine (e.g., 30% is the target percent) then the nucleotide mixture can contain 7.5% deoxyinosine (e.g., 30/4 = 7.5%), 17.5% dGTP, and 25% each for dATP, dCTP and dTTP. The target percent of dGTP to be replaced by deoxyinosine can be about 0.1-1%, or about 1-5%, or about 5-10%, or about 10-20%, or about 20-30% , or about 30-45%, or about 45-50%, or a higher percent of the dGTP in the immobilized concatemer template molecules are replaced with nucleotides having a scissile moiety.

[00380] In some embodiments, the nucleotide mixture can include an amount of 8oxoG so that a target percent of the guanosine in the resulting concatemer molecules are replaced with 8oxoG. For example, when 30% of dGTP in the concatemer molecules are to be replaced with 8oxoG (e.g., 30% is the target percent) then the nucleotide mixture can contain 7.5% 8oxoG (e.g., 30/4 = 7.5%), 17.5% dGTP, and 25% each for dATP, dCTP and dTTP. The target percent of dGTP to be replaced by 8oxoG can be about 0.1-1%, or about 1-5%, or about 5-10%, or about 10-20%, or about 20-30% , or about 30-45%, or about 45-50%, or a higher percent of the dGTP in the immobilized concatemer template molecules are replaced with nucleotides having a scissile moiety.

[00381] In some embodiments, the rolling circle amplification reaction generates immobilized concatemer template molecules with incorporated nucleotides having a scissile moiety that are distributed at random positions along individual immobilized concatemer template molecules. In some embodiments, the nucleotides having a scissile moiety are distributed at different positions in the different immobilized concatemer template molecules.

[00382] In some embodiments, the pairwise sequencing method further comprises **step (c):** sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands. The sequencing of step (c) comprises contacting the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers under a condition suitable to hybridize at least one forward sequencing primer to at least one of the forward sequencing primer binding sites/sequences of the immobilized concatemer template molecules, and conducting forward sequencing reactions using one or more types of sequencing polymerases, a plurality of nucleotides and/or multivalent

molecules, and the hybridized first forward sequencing primers (Figure 27). In some embodiments, the soluble forward sequencing primers comprise 3' OH extendible ends. In some embodiments, the soluble forward sequencing primers comprise a 3' blocking moiety which can be removed to generate a 3' OH extendible end. In some embodiments, the soluble forward sequencing primers lack a nucleotide having a scissile moiety. The forward sequencing reactions can generate a plurality of extended forward sequencing primer strands. In some embodiments, individual immobilized concatemer template molecules have multiple copies of the forward sequencing primer binding sites, wherein each forward sequencing primer binding site is capable of hybridizing to a first forward sequencing primer. Individual forward sequencing primer binding sites in a given immobilized concatemer template molecule can be hybridized to a forward sequencing primer and can undergo a sequencing reaction. Individual immobilized concatemer template molecules can undergo two or more sequence reactions, where each sequencing reaction is initiated from a first forward sequencing primer that is hybridized to a forward sequencing primer binding site (e.g., see Figure 27). In some embodiments, the sequencing reactions comprise a plurality of nucleotides (or analogs thereof) labeled with a detectable reporter moiety. In some embodiments, the sequencing reaction comprise a plurality of multivalent molecules having a plurality of nucleotide units attached to a core, where the multivalent molecules are labeled with a detectable reporter moiety. In some embodiments, the core is labeled with a detectable reporter moiety. In some embodiments, at least one linker and/or at least one nucleotide unit of a nucleotide arm is labeled with a detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore. An exemplary nucleotide arm is shown in Figure 108, and exemplary multivalent molecules are shown in Figures 104-107.

[00383] In some embodiments, the pairwise sequencing method further comprises **step (d):** retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules. The plurality of extended forward sequencing primer strands can be removed and replaced with a plurality of forward extension strands by conducting a primer extension reaction (see Figures 28-30).

In some embodiments, step (d) comprises contacting at least one extended forward sequencing primer strand with a plurality of strand displacing polymerases and a plurality of nucleotides and in the absence of soluble amplification primers, under a condition suitable to conduct a strand displacing primer extension reaction using the at least one extended forward sequencing primers strand to initiate the primer extension reaction thereby generating a forward extension strand that is covalently joined to the extended forward sequencing primers strand, wherein the forward extension strand is hybridized to the immobilized concatemer template molecule (Figure 28). For example, one of the extended forward sequencing primer strands can serve as a primer for the strand displacing polymerase. The strand displacing polymerase can extend the extended forward sequencing primer strand, and displace downstream extended forward sequencing primer strands while synthesizing an extended strand that replaces the downstream extended forward sequencing primer strands. The newly extended strand is covalently joined to an extended forward sequencing primer strand. The immobilized concatemer template molecules are retained. The primer extension reaction can optionally include a plurality of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) to generate forward extension strands. Individual forward extension strands can collapse into a nanoball having a more compact size and/or shape compared to a nanoball generated from a primer extension reaction conducted without compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III). Inclusion of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) in the primer extension reaction can improve FWHM (full width half maximum) of a spot image of the nanoball. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

[00384] Examples of strand displacing polymerases include phi29 DNA polymerase, large fragment of Bst DNA polymerase, large fragment of Bsu DNA polymerase (exo-), Bca DNA polymerase (exo-), Klenow fragment of E. coli DNA polymerase, T5 polymerase, M-MuLV reverse transcriptase, HIV viral reverse transcriptase, Deep Vent DNA polymerase and KOD DNA polymerase. The phi29 DNA polymerase can be wild type phi29 DNA polymerase (e.g., MagniPhi from Expedeon), or variant EquiPhi29 DNA polymerase (e.g., from Thermo Fisher Scientific), or chimeric QualiPhi DNA polymerase (e.g., from 4basebio).

[00385] In some embodiments, step (d) comprises: **(i)** removing the plurality of extended forward sequencing primer strand while retaining the immobilized concatemer template molecules; and **(ii)** contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble forward sequencing primers (e.g., a second plurality of soluble forward sequencing primers), a plurality of nucleotides (e.g., a second plurality of nucleotides) and a plurality of primer extension polymerases, under a condition suitable to hybridize the plurality of soluble forward sequencing primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed primer extension reactions thereby generating a plurality of forward extension strands, wherein the soluble sequencing primers hybridize with the forward sequencing primer binding sequence in the retained immobilized concatemer molecules (Figure 29). The primer extension reaction can optionally include a plurality of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) to generate forward extension strands. Individual forward extension strands can collapse into a nanoball having a more compact size and/or shape compared to a nanoball generated from a primer extension reaction conducted without compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III). Inclusion of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) in the primer extension reaction can improve FWHM (full width half maximum) of a spot image of the nanoball. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

[00386] In some embodiments, in step (d), the condition suitable to hybridize the plurality of soluble forward sequencing primers to the plurality of retained immobilized single stranded nucleic acid concatemer template molecules comprises hybridizing retained immobilized concatemer template molecules with the soluble primers in the presence of a primer extension polymerase, a plurality of nucleotides, and a high efficiency hybridization buffer. In some embodiment, the high efficiency hybridization buffer comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer

formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

[00387] In some embodiments, step (d) comprises: **(i)** removing the plurality of extended forward sequencing primer strand while retaining the immobilized concatemer template molecules; and **(ii)** contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble amplification primers, a plurality of nucleotides (e.g., a second plurality of nucleotides) and a plurality of primer extension polymerases, under a condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed primer extension reactions thereby generating a plurality of forward extension strands, wherein the soluble amplification primers hybridize with the soluble amplification primer binding sequence in the retained immobilized concatemer molecules (Figure 30). The primer extension reaction can optionally include a plurality of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) to generate forward extension strands. Individual forward extension strands can collapse into a nanoball having a more compact size and/or shape compared to a nanoball generated from a primer extension reaction conducted without compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III). Inclusion of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) in the primer extension reaction can improve FWHM (full width half maximum) of a spot image of the nanoball. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

[00388] In some embodiments, in step (d), the condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized single stranded nucleic acid concatemer template molecules comprises hybridizing retained immobilized concatemer

template molecules with the soluble primers in the presence of a primer extension polymerase, a plurality of nucleotides, and a high efficiency hybridization buffer. In some embodiment, the high efficiency hybridization buffer comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

[00389] In some embodiments, in step (d), the plurality of extended forward sequencing primer strands can be removed using an enzyme or a chemical reagent. For example, the plurality of extended forward sequencing primer strands can be enzymatically degraded using a 5' to 3' double-stranded DNA exonuclease, including T7 exonuclease (e.g., from New England Biolabs, catalog # M0263S). In some embodiments, the plurality of extended forward sequencing primer strands can be removed with a temperature that favors nucleic acid denaturation.

[00390] In some embodiments, in step (d), a denaturation reagent can be used to remove the plurality of extended forward sequencing primer strands, wherein the denaturation reagent comprises any one or any combination of compounds such as formamide, acetonitrile, guanidinium chloride and/or a buffering agent (e.g., Tris-HCl, MES, HEPES, or the like).

[00391] In some embodiments, in step (d), the plurality of extended forward sequencing primer strands can be removed using an elevated temperature (e.g., heat) with or without a nucleic acid denaturation reagent. The plurality of extended forward sequencing primer strands can be subjected to a temperature of about 45-50 °C, or about 50-60 °C, or about 60-70 °C, or about 70-80 °C, or about 80-90 °C, or about 90-95 °C, or higher temperature.

[00392] In some embodiments, in step (d), the plurality of extended forward sequencing primer strands can be removed using 100% formamide at a temperature of about 65 °C for about 3 minutes, and washing with a reagent comprising about 50 mM NaCl or equivalent ionic strength and having a pH of about 6.5 – 8.5.

[00393] In some embodiments, the primer extension polymerase of step (d) comprises a high fidelity polymerase. In some embodiments, the primer extension polymerase of step (d) comprises a DNA polymerase capable of catalyzing a primer extension reaction using a uracil-containing template molecule (e.g., a uracil-tolerant polymerase). Exemplary polymerases include, but are not limited to, Q5U Hot Start high-fidelity DNA polymerase (e.g., catalog # M0515S from New England Biolabs), Taq DNA polymerase, One Taq DNA polymerase (e.g., mixture of Taq and Deep Vent DNA polymerases, catalog #M0480S from New England Biolabs), LongAmp Taq DNA polymerase (e.g., catalog #M0323S from New England Biolabs), Epimark Hot Start Taq DNA polymerase (e.g., catalog #M0490S from New England Biolabs), Bst DNA polymerase (e.g., large fragment, catalog #M0275S from New England Biolabs), Bsu DNA polymerase (e.g., large fragment, catalog #M0330S from New England Biolabs), Phi29 DNA polymerase (e.g., catalog # M0269S from New England Biolabs), *E. coli* DNA polymerase (e.g., catalog # M0209S from New England Biolabs), Therminator DNA polymerase (e.g., catalog #M0261S from New England Biolabs), Vent DNA polymerase and Deep Vent DNA polymerase.

[00394] The pairwise methods described herein can provide increased accuracy in a downstream sequencing reaction because step (d) replaces the extended forward sequencing primer strands that were generated in step (c) with forward extension strands having reduced base errors. The extended forward sequencing primer strands are generated in step (c) and may or may not contain erroneously incorporated nucleotides due to polymerase-catalyzed mis-paired bases. When step (d) is conducted with a high fidelity DNA polymerase, the resulting forward extension strands may have reduced base errors compared to the extended forward sequencing primer strands. The forward extension strands will be used as a nucleic acid template for a downstream sequencing step (e.g., see step (f) below). Thus, step (d) can increase the sequencing accuracy of the downstream step (f) and therefore increase the overall sequencing accuracy of the pairwise sequencing workflow.

[00395] In some embodiments, the pairwise sequencing method further comprises **step (e):** removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized surface primers (Figures 31 and 33).

[00396] The abasic sites are generated on the retained concatemer template strands that contain nucleotides having scissile moieties. In some embodiments, the scissile moieties in the retained concatemer template molecules comprises uridine, 8-oxo-7,8-dihydroguanine (e.g., 8oxoG) or deoxyinosine. The abasic sites can be removed to generate a plurality of single stranded nucleic acid template molecules having gaps while retaining the plurality of forward extension strands. The abasic sites can be generated by contacting the immobilized concatemer template molecules with an enzyme that removes the nucleo-base at the nucleotide having the scissile moiety. The uracil in the retained concatemer template strands can be converted to an abasic site using uracil DNA glycosylase (UDG). The 8oxoG in the retained concatemer template strands can be converted to an abasic site using FPG glycosylase. The deoxyinosine in the retained concatemer template strands can be converted to an abasic site using AlkA glycosylase.

[00397] In some embodiments, in step (e), the gaps can be generated by contacting the abasic sites in the immobilized concatemer template molecules with an enzyme or a mixture of enzymes having lyase activity that breaks the phosphodiester backbone at the 5' and 3' sides of the abasic site to release the base-free deoxyribose and generate a gap (Figures 31 and 33). The abasic sites can be removed using AP lyase, Endo IV endonuclease, FPG glycosylase/AP lyase, Endo VIII glycosylase/AP lyase. In some embodiments, generating the abasic sites and removal of the abasic sites to generate gaps can be achieved using a mixture of uracil DNA glycosylase and DNA glycosylase-lyase endonuclease VIII, for example USER (Uracil-Specific Excision Reagent Enzyme from New England Biolabs) or thermolabile USER (also from New England Biolabs).

[00398] In some embodiments, in step (e), the plurality of gap-containing template molecules can be removed using an enzyme, chemical compound and/or heat. After the gap-removal

procedure, the plurality of retained forward extension strands are hybridized to the retained immobilized surface primers (Figures 32 and 34).

[00399] For example, the plurality of gap-containing template molecules can be enzymatically degraded using a 5' to 3' double-stranded DNA exonuclease, including T7 exonuclease (e.g., from New England Biolabs, catalog # M0263S). When a 5' to 3' double-stranded DNA exonuclease is used for removing gap-containing template molecules, then the plurality of soluble amplification primers in step (e) can comprise at least one phosphorothioate diester bond at their 5' ends which can render the soluble amplification primers resistant to exonuclease degradation. In some embodiments, the plurality of soluble amplification primers in step (d) comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5' ends. In some embodiments, the plurality soluble amplification primers in step (d) comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the forward sequencing primers resistant to exonuclease degradation.

[00400] In some embodiments, the plurality of gap-containing template molecules can be removed using a chemical reagent that favors nucleic acid denaturation. The denaturation reagent can include any one or any combination of compounds such as formamide, acetonitrile, guanidinium chloride and/or a buffering agent (e.g., Tris-HCl, MES, HEPES, or the like).

[00401] In some embodiments, the plurality of gap-containing template molecules can be removed using an elevated temperature (e.g., heat) with or without a nucleic acid denaturation reagent. The gap-containing template molecules can be subjected to a temperature of about 45-50 °C, or about 50-60 °C, or about 60-70 °C, or about 70-80 °C, or about 80-90 °C, or about 90-95 °C, or higher temperature.

[00402] In some embodiments, the plurality of gap-containing template molecules can be removed using 100% formamide at a temperature of about 65 °C for about 3 minutes, and washing with a reagent comprising about 50 mM NaCl or equivalent ionic strength and having a pH of about 6.5 – 8.5.

[00403] In some embodiments, the pairwise sequencing method further comprises **step (f):** sequencing the plurality of retained forward extension strands thereby generating a plurality of extended reverse sequencing primer strands. In some embodiments, the sequencing of step (f) comprises contacting the plurality of retained forward extension strands with a plurality of soluble reverse sequencing primers under a condition suitable to hybridize the reverse

sequencing primers to the reverse sequencing primer binding site of the retained forward extension strands, and by conducting sequencing reactions using the hybridized reverse sequencing primers wherein the forward sequencing reactions generates a plurality of extended reverse sequencing primer strands (Figures 35 and 36). The extended reverse sequencing primer strands are hybridized to the retained forward extension strand. The retained forward extension strand is hybridized to the first surface primer. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support.

[00404] For the sake of simplicity, Figures 32 and 34 show exemplary retained forward extension strands each having one copy of the sequence of interest and various universal primer binding sites. The skilled artisan will appreciate that the retained forward extension strand can include two or more tandem copies containing the sequence of interest and various universal primer binding sites. Therefore, the reverse sequencing reaction can generate a plurality of extended reverse sequencing primer strands hybridized to the same retained forward extension strand.

[00405] In some embodiments, in step (f), the condition suitable to hybridize the reverse sequencing primers to the reverse sequencing primer binding sequences of the retained forward extension strands comprises contacting the plurality of soluble reverse sequencing primers and the retained forward extension strands with a high efficiency hybridization buffer. In some embodiments, the high efficiency hybridization buffer comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises

polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

[00406] In an alternative embodiment, the sequencing of step (f) comprises using the immobilized surface primer as a sequencing primer and conducting sequencing reactions to generate a plurality of reverse sequencing strands.

[00407] In some embodiments, the reverse sequencing reactions of step (f) comprises contacting the plurality of reverse sequencing primers with the reverse sequencing primer binding sequences of the retained forward extension strands, one or more types of sequencing polymerases, and a plurality of nucleotides and/or a plurality of multivalent molecules. In some embodiments, the soluble reverse sequencing primers comprise 3' OH extendible ends. In some embodiments, the soluble reverse sequencing primers comprise a 3' blocking moiety which can be removed to generate a 3' OH extendible end. In some embodiments, the soluble reverse sequencing primers lack a nucleotide having a scissile moiety. The sequencing reactions that employ nucleotides and/or multivalent molecules is described in more detail below. The reverse sequencing reactions can generate a plurality of extended reverse sequencing primer strands. In some embodiments, individual retained forward extension strands have multiple copies of the reverse sequencing primer binding sequences/sites, wherein each reverse sequencing primer binding site is capable of hybridizing to a reverse sequencing primer. Individual reverse sequencing primer binding sites in a given retained forward extension strand can be hybridized to a reverse sequencing primer and can undergo a sequencing reaction. Thus, an individual retained forward extension strand can undergo two or more sequence reactions, where each sequencing reaction is initiated from a reverse sequencing primer that is hybridized to a reverse sequencing primer binding site (e.g., see Figures 35 and 36). In some embodiments, the sequencing reactions comprise a plurality of nucleotides (or analogs thereof) labeled with a detectable reporter moiety. In some embodiments, the sequencing reaction comprise a plurality of multivalent molecules having nucleotide units, where the multivalent molecules are labeled with a detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore.

[00408] In some embodiments, at least one washing step can be conducted after any of steps (a) – (f). The washing step can be conducted with a wash buffer comprising a pH buffering agent, a metal chelating agent, a salt, and a detergent.

[00409] In some embodiments, the pH buffering compound in the wash buffer comprises any one or any combination of two or more of Tris, Tris-HCl, Tricine, Bicine, Bis-Tris propane, HEPES, MES, MOPS, MOPSO, BES, TES, CAPS, TAPS, TAPSO, ACES, PIPES, ethanolamine (a.k.a 2-amino methanol; MEA), a citrate compound, a citrate mixture, NaOH and/or KOH. In some embodiments, the pH buffering agent can be present in the wash buffer at a concentration of about 1-100 mM, or about 10-50 mM, or about 10-25 mM. In some embodiments, the pH of the pH buffering agent which is present in any of the reagents described here in can be adjusted to a pH of about 4-9, or a pH of about 5-9, or a pH of about 5-8.

[00410] In some embodiments, the metal chelating agent in the wash buffer comprises EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol tetraacetic acid), HEDTA (hydroxyethylethylenediaminetriacetic acid), DPTA (diethylene triamine pentaacetic acid), NTA (N,N-bis(carboxymethyl)glycine), citrate anhydrous, sodium citrate, calcium citrate, ammonium citrate, ammonium bicitrate, citric acid, potassium citrate, or magnesium citrate. In some embodiments, the wash buffer comprises a chelating agent at a concentration of about 0.01 – 50 mM, or about 0.1 – 20 mM, or about 0.2 – 10 mM.

[00411] In some embodiments, the salt in the wash buffer comprises NaCl, KCl, NH_2SO_4 or potassium glutamate. In some embodiments, the detergent comprises an ionic detergent such as SDS (sodium dodecyl sulfate). The wash buffer can include a monovalent salt at a concentration of about 25-500 mM, or about 50-250 mM, or about 100-200 mM.

[00412] In some embodiments, the detergent in the wash buffer comprises a non-ionic detergent such as Triton X-100, Tween 20, Tween 80 or Nonidet P-40. In some embodiments, the detergent comprises a zwitterionic detergent such as CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) or *N*-Dodecyl-*N,N*-dimethyl-3-amonio-1-propanesulfate (DetX). In some embodiments, the detergent comprises LDS (lithium dodecyl sulfate), sodium taurodeoxycholate, sodium taurocholate, sodium glycocholate, sodium deoxycholate or sodium cholate. In some embodiments, the detergent is included in the wash buffer at a concentration of about 0.01-0.05%, or about 0.05-0.1%, or about 0.1-0.15%, or about 0.15-0.2%, or about 0.2-0.25%.

In Solution RCA and Pairwise Sequencing – Generating Abasic Sites

[00413] The present disclosure provides pairwise sequencing methods, comprising **step (a):** contacting in-solution a plurality of single-stranded circular nucleic acid library molecules to a

plurality of soluble first amplification primers, a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety, under a condition suitable to form a plurality of library-primer duplexes and suitable for conducting a rolling circle amplification reaction, thereby generating a plurality of single stranded nucleic acid concatemers having at least one nucleotide with a scissile moiety (Figure 38). In some embodiments, the soluble first amplification primer comprises a sequence that selectively hybridizes to a universal binding sequence in the circular nucleic acid library molecules, such as for example a universal binding sequence (or a complementary sequence thereof) for the first soluble amplification primer. Alternatively, the soluble first amplification primer comprises a random sequence that binds non-selectively to a sequence in the circular nucleic acid library molecules.

[00414] In some embodiments, individual single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest and wherein the individual library molecules further comprise any one or any combination of two or more of **(i)** a universal binding sequence (or a complementary sequence thereof) for a soluble forward sequencing primer, **(ii)** a universal binding sequence (or a complementary sequence thereof) for a soluble reverse sequencing primer, **(iii)** a universal binding sequence (or a complementary sequence thereof) for an immobilized first surface primer, **(iv)** a universal binding sequence (or a complementary sequence thereof) for an immobilized second surface primer, **(v)** a universal binding sequence (or a complementary sequence thereof) for a first soluble amplification primer, **(vi)** a universal binding sequence (or a complementary sequence thereof) for a second soluble amplification primer, **(vii)** a universal binding sequence (or a complementary sequence thereof) for a soluble compaction oligonucleotide, **(viii)** a sample barcode sequence and/or **(ix)** a unique molecular index sequence. In some embodiments, the single-stranded circular nucleic acid library molecules comprise covalently closed circular molecules.

[00415] In some embodiments, the rolling circle amplification reaction of step (a) generates a plurality of single stranded nucleic acid concatemer molecules in solution, comprising a concatemer having at least one nucleotide having a scissile moiety. In some embodiments, individual concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of: (i) two or more copies of a

universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, (viii) two or more copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[00416] In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the forward sequencing primer can hybridize to at least a portion of the forward sequencing primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the reverse sequencing primer can hybridize to at least a portion of the reverse sequencing primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the immobilized first surface primer can hybridize to at least a portion of the immobilized first surface primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the immobilized second surface primer can hybridize to at least a portion of the immobilized second surface primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the first soluble amplification primer can hybridize to at least a portion of the first soluble amplification primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the second soluble amplification primer can hybridize to at least a portion of the second soluble amplification primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the soluble compaction oligonucleotide can hybridize to at least a portion of the soluble compaction oligonucleotide.

[00417] The in-solution rolling circle amplification reaction of step (a) can be conducted with a nucleotide mixture containing dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety to generate the concatemer molecules which includes at least one nucleotide having a scissile moiety. The scissile moieties in the concatemer molecules can be converted into abasic sites. In some embodiments, in the nucleotide mixture, the nucleotide having the scissile moiety comprises uridine, 8-oxo-7,8-dihydroguanine (e.g., 8oxoG) or deoxyinosine. In the concatemer

molecules, the uridine can be converted to an abasic site using uracil DNA glycosylase (UDG), the 8oxoG can be converted to an abasic site using FPG glycosylase, and the deoxyinosine can be converted to an abasic site using AlkA glycosylase.

[00418] In some embodiments, the nucleotide mixture can include an amount of dUTP so that a target percent of the thymidine in the resulting concatemer molecules are replaced with dUTP. For example, when 30% of dTTP in the concatemer molecules are to be replaced with dUTP (e.g., 30% is the target percent) then the nucleotide mixture can contain 7.5% dUTP (e.g., 30/4 = 7.5%), 17.5% dTTP, and 25% each for dATP, dCTP and dGTP. The target percent of dTTP to be replaced by dUTP can be about 0.1-1%, or about 1-5%, or about 5-10%, or about 10-20%, or about 20-30% , or about 30-45%, or about 45-50%, or a higher percent of the dTTP in the concatemer molecules are replaced with nucleotides having a scissile moiety.

[00419] In some embodiments, the nucleotide mixture can include an amount of deoxyinosine so that a target percent of the guanosine in the resulting concatemer molecules are replaced with deoxyinosine. For example, when 30% of dGTP in the concatemer molecules are to be replaced with deoxyinosine (e.g., 30% is the target percent) then the nucleotide mixture can contain 7.5% deoxyinosine (e.g., 30/4 = 7.5%), 17.5% dGTP, and 25% each for dATP, dCTP and dTTP. The target percent of dGTP to be replaced by deoxyinosine can be about 0.1-1%, or about 1-5%, or about 5-10%, or about 10-20%, or about 20-30% , or about 30-45%, or about 45-50%, or a higher percent of the dGTP in the concatemer molecules are replaced with nucleotides having a scissile moiety.

[00420] In some embodiments, the nucleotide mixture can include an amount of 8oxoG so that a target percent of the guanosine in the resulting concatemer molecules are replaced with 8oxoG. For example, when 30% of dGTP in the concatemer molecules are to be replaced with 8oxoG (e.g., 30% is the target percent) then the nucleotide mixture can contain 7.5% 8oxoG (e.g., 30/4 = 7.5%), 17.5% dGTP, and 25% each for dATP, dCTP and dTTP. The target percent of dGTP to be replaced by 8oxoG can be about 0.1-1%, or about 1-5%, or about 5-10%, or about 10-20%, or about 20-30% , or about 30-45%, or about 45-50%, or a higher percent of the dGTP in the concatemer molecules are replaced with nucleotides having a scissile moiety.

[00421] In some embodiments, the in-solution rolling circle amplification reaction generates concatemer molecules with incorporated nucleotides having a scissile moiety that are distributed at random positions along individual immobilized concatemer template molecules. In some

embodiments, the nucleotides having a scissile moiety are distributed at different positions in the different concatemer molecules.

[00422] In some embodiments, the pairwise sequencing method further comprises **step (b):** distributing the rolling circle amplification reaction from step (a) onto a support having a plurality of the first surface primers immobilized thereon, under a condition suitable for hybridizing one or more portions of individual single stranded concatemers to one or more immobilized first surface primers (Figure 39). In some embodiments, the immobilized first surface primers have terminal 3' group that are non-extendible. In some embodiments, the 3' terminal end of the immobilized first surface primers comprise a moiety that blocks primer extension, such as for example a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group. In some embodiments, the immobilized first surface primer have an extendible 3'OH end. In some embodiments, the immobilized first surface primers lack a nucleotide having a scissile moiety. The concatemers are immobilized to the support by hybridization to the immobilized first surface primers. In some embodiments, the support comprises a plurality of first surface primers. In some embodiments, the support lacks a plurality of second surface primers. In some embodiments, the support comprises a plurality of first and second surface primers.

[00423] In some embodiments, the pairwise sequencing method further comprises **step (c):** continuing the rolling circle amplification reaction on the support to generate a plurality of extended concatemer template molecules that are immobilized via hybridization to the immobilized first surface primers (Figure 40). The on-support RCA reaction can be conducted with a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety, under a condition suitable to generate a plurality of extended concatemers having at least one nucleotide with a scissile moiety (Figure 41). In some embodiments, the rolling circle amplification reaction on the support can be conducted in the presence, or in the absence, of a plurality of compaction oligonucleotides.

[00424] In some embodiments, the on-support rolling circle amplification reaction generates immobilized concatemer template molecules with incorporated nucleotides having a scissile moiety that are distributed at random positions along individual immobilized concatemer

template molecules. In some embodiments, the nucleotides having a scissile moiety are distributed at different positions in the different immobilized concatemer template molecules.

[00425] In some embodiments, the immobilized first surface primers comprise single stranded oligonucleotides comprising DNA, RNA or a combination of DNA and RNA. The first surface primers comprise a sequence that is wholly complementary or partially complementary along their lengths to at least a portion of the concatemer molecules. In some embodiments, the first surface primers can lack a terminal 3' OH extendible end which renders the first surface primers non-extendible. In some embodiments, the first surface primers include a terminal 3' OH group which is extendible for nucleotide polymerization (e.g., polymerase catalyzed polymerization). The immobilized first surface primers can be immobilized to the support or immobilized to a coating on the support. The immobilized first surface primers can be embedded and attached (coupled) to the coating on the support. In some embodiments, the 5' end of the immobilized first surface primers are immobilized to a support or immobilized to a coating on the support. Alternatively, an interior portion or the 3' end of the immobilized first surface primers can be immobilized to a support or immobilized to a coating on the support. The support comprises a plurality of immobilized first surface primers having the same sequence. The immobilized first surface primers can be any length, for example 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths.

[00426] In some embodiments, the plurality of immobilized first surface primers comprise 3' extendible ends. In some embodiments, the 3' terminal end of the immobilized first surface primers comprise a moiety that blocks primer extension, such as for example a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group. In some embodiments, the immobilized first surface primers are not extendible in a primer extension reaction. The immobilized first surface primers lack a nucleotide having a scissile moiety.

[00427] In some embodiments, the plurality of immobilized first surface primers comprise at least one phosphorothioate diester bond at their 5' ends which can render the first surface primers resistant to exonuclease degradation. In some embodiments, the plurality of immobilized first surface primers comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5' ends. In some embodiments, the plurality of immobilized first surface primers comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the first surface primers resistant to exonuclease degradation.

[00428] In some embodiments, the immobilized first surface primers comprise at least one locked nucleic acid (LNA) which comprises a methylene bridge bond between a 2' oxygen and 4' carbon of the pentose ring. Immobilized first surface primers that include at least one LNA can be resistant to nuclease digestions and can exhibit increased melting temperature when hybridized to the concatemer template molecules.

[00429] In some embodiments, the support further comprises a plurality of a second surface primer immobilized thereon (Figure 52). The second surface primers have a sequence that differs from the first immobilized surface primer. The immobilized second surface primers comprise single stranded oligonucleotides comprising DNA, RNA or a combination of DNA and RNA. The second surface primers comprise a sequence that is wholly complementary or partially complementary along their lengths to at least a portion of a concatemer molecule. The immobilized second surface primers can be immobilized to the support or immobilized to a coating on the support. The immobilized second surface primers can be embedded and attached (coupled) to the coating on the support. In some embodiments, the 5' end of the immobilized second surface primers are immobilized to a support or immobilized to a coating on the support. Alternatively, an interior portion or the 3' end of the immobilized second surface primers can be immobilized to a support or immobilized to a coating on the support. The support comprises a plurality of immobilized second surface primers having the same sequence. The immobilized second surface primers can be any length, for example 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths.

[00430] In some embodiments, the 3' terminal end of the immobilized second surface primers comprise an extendible 3' OH moiety. In some embodiments, the 3' terminal end of the immobilized second surface primers comprise a 3' non-extendible moiety. The 3' terminal end of the immobilized second surface primers comprise a moiety that blocks primer extension, such as for example a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group. The immobilized second surface primers are not extendible in a primer extension reaction. The immobilized second surface primers lack a nucleotide having a scissile moiety.

[00431] In some embodiments, the plurality of immobilized second surface primers comprise at least one phosphorothioate diester bond at their 5' ends which can render the second surface primers resistant to exonuclease degradation. In some embodiments, the plurality of immobilized second surface primers comprise 2-5 or more consecutive phosphorothioate diester bonds at their

5' ends. In some embodiments, the plurality of immobilized second surface primers comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the second surface primers resistant to exonuclease degradation.

[00432] In some embodiments, individual immobilized single stranded nucleic acid concatemer template molecule are hybridized to an immobilized first surface primer, and at least one portion of the individual concatemer template molecule is hybridized to an immobilized second surface primer (Figure 52). The immobilized second surface primers serve to pin down a portion of the immobilized concatemer template molecules to the support. The immobilized concatemer template molecule has two or more copies of a universal binding sequence for an immobilized second surface primer. The portion of the immobilized concatemer template molecule that includes the universal binding sequence for an immobilized second surface primer can hybridize to the immobilized second surface primer. In some embodiments, the second surface primers include a terminal 3' blocking group that renders them non-extendible. In some embodiments, the second surface primers have terminal 3' extendible ends.

[00433] In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized first surface primers per mm^2. In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized second surface primers per mm^2. In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized first surface primers and immobilized second surface primers per mm^2.

[00434] The immobilized surface primers (e.g., first and second surface primers) are in fluid communication with each other to permit flowing various solutions of linear or circular nucleic acid template molecules, soluble primers, enzymes, nucleotides, divalent cations, buffers, reagents, and the like, onto the support so that the plurality of immobilized surface primers react with the solutions in a massively parallel manner.

[00435] In some embodiments, the pairwise sequencing method further comprises **step (d):** sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands. The sequencing of step (d) comprises contacting the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers under a condition suitable to hybridize at least one forward sequencing primer to at least one of the forward sequencing primer binding sites/sequences of the immobilized concatemer template molecules, and conducting forward sequencing reactions

using one or more types of sequencing polymerases, a plurality of nucleotides and/or multivalent molecules, and the hybridized first forward sequencing primers. In some embodiments, the soluble forward sequencing primers comprise 3' OH extendible ends. In some embodiments, the soluble forward sequencing primers comprise a 3' blocking moiety which can be removed to generate a 3' OH extendible end. In some embodiments, the soluble forward sequencing primers lack a nucleotide having a scissile moiety. The forward sequencing reactions can generate a plurality of extended forward sequencing primer strands (Figure 42). In some embodiments, individual immobilized concatemer template molecules have multiple copies of the forward sequencing primer binding sites, wherein each forward sequencing primer binding site is capable of hybridizing to a first forward sequencing primer. Individual forward sequencing primer binding sites in a given immobilized concatemer template molecule can be hybridized to a forward sequencing primer and can undergo a sequencing reaction. Individual immobilized concatemer template molecules can undergo two or more sequence reactions, where each sequencing reaction is initiated from a first forward sequencing primer that is hybridized to a forward sequencing primer binding site (e.g., see Figure 42). In some embodiments, the sequencing reactions comprise a plurality of nucleotides (or analogs thereof) labeled with a detectable reporter moiety. In some embodiments, the sequencing reaction comprise a plurality of multivalent molecules having a plurality of nucleotide units attached to a core, where the multivalent molecules are labeled with a detectable reporter moiety. In some embodiments, the core is labeled with a detectable reporter moiety. In some embodiments, at least one linker and/or at least one nucleotide unit of a nucleotide arm is labeled with a detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore. An exemplary nucleotide arm is shown in Figure 108, and exemplary multivalent molecules are shown in Figures 104-107.

[00436] In some embodiments, the pairwise sequencing method further comprises **step (e):** retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules. The plurality of extended forward sequencing primer strands can be removed and replaced with a plurality of forward extension strands by conducting a primer extension reaction (See Figures 43-45).

[00437] In some embodiments, step (e) comprises contacting at least one extended forward sequencing primer strand with a plurality of strand displacing polymerases and a plurality of nucleotides and in the absence of soluble amplification primers, under a condition suitable to conduct a strand displacing primer extension reaction using the at least one extended forward sequencing primers strand to initiate the primer extension reaction thereby generating a forward extension strand that is covalently joined to the extended forward sequencing primers strand, wherein the forward extension strand is hybridized to the immobilized concatemer template molecule (Figure 43). For example, one of the extended forward sequencing primer strands can serve as a primer for the strand displacing polymerase. The strand displacing polymerase can extend the extended forward sequencing primer strand, and displace downstream extended forward sequencing primer strands while synthesizing an extended strand that replaces the downstream extended forward sequencing primer strands. The newly extended strand is covalently joined to an extended forward sequencing primer strand. The immobilized concatemer template molecules are retained. The primer extension reaction can optionally include a plurality of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) to generate forward extension strands. Individual forward extension strands can collapse into a nanoball having a more compact size and/or shape compared to a nanoball generated from a primer extension reaction conducted without compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III). Inclusion of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) in the primer extension reaction can improve FWHM (full width half maximum) of a spot image of the nanoball. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

[00438] Examples of strand displacing polymerases include phi29 DNA polymerase, large fragment of Bst DNA polymerase, large fragment of Bsu DNA polymerase (exo-), Bca DNA polymerase (exo-), Klenow fragment of E. coli DNA polymerase, T5 polymerase, M-MuLV reverse transcriptase, HIV viral reverse transcriptase, Deep Vent DNA polymerase and KOD DNA polymerase. The phi29 DNA polymerase can be wild type phi29 DNA polymerase (e.g., MagniPhi from Expedeon), or variant EquiPhi29 DNA polymerase (e.g., from Thermo Fisher Scientific), or chimeric QualiPhi DNA polymerase (e.g., from 4basebio).

[00439] In some embodiments, step (e) comprises: **(i)** removing the plurality of extended forward sequencing primer strand while retaining the immobilized concatemer template molecules; and **(ii)** contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble forward sequencing primers (e.g., a second plurality of soluble forward sequencing primers), a plurality of nucleotides (e.g., a second plurality of nucleotides) and a plurality of primer extension polymerases, under a condition suitable to hybridize the plurality of soluble forward sequencing primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed primer extension reactions thereby generating a plurality of forward extension strands, wherein the soluble sequencing primers hybridize with the forward sequencing primer binding sequence in the retained immobilized concatemer molecules (Figure 44). The primer extension reaction can optionally include a plurality of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) to generate forward extension strands. Individual forward extension strands can collapse into a nanoball having a more compact size and/or shape compared to a nanoball generated from a primer extension reaction conducted without compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III). Inclusion of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) in the primer extension reaction can improve FWHM (full width half maximum) of a spot image of the nanoball. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

[00440] In some embodiments, in step (e), the condition suitable to hybridize the plurality of soluble forward sequencing primers to the plurality of retained immobilized single stranded nucleic acid concatemer template molecules comprises hybridizing retained immobilized concatemer template molecules with the soluble primers in the presence of a primer extension polymerase, a plurality of nucleotides, and a high efficiency hybridization buffer. In some embodiment, the high efficiency hybridization buffer comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer

formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

[00441] In some embodiments, step (e) comprises: **(i)** removing the plurality of extended forward sequencing primer strand while retaining the immobilized concatemer template molecules; and **(ii)** contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble amplification primers, a plurality of nucleotides (e.g., a second plurality of nucleotides) and a plurality of primer extension polymerases, under a condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed primer extension reactions thereby generating a plurality of forward extension strands, wherein the soluble amplification primers hybridize with the soluble amplification primer binding sequence in the retained immobilized concatemer molecules (Figure 45). The primer extension reaction can optionally include a plurality of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) to generate forward extension strands. Individual forward extension strands can collapse into a nanoball having a more compact size and/or shape compared to a nanoball generated from a primer extension reaction conducted without compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III). Inclusion of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) in the primer extension reaction can improve FWHM (full width half maximum) of a spot image of the nanoball. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

[00442] In some embodiments, in step (e), the condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized single stranded nucleic acid concatemer template molecules comprises hybridizing retained immobilized concatemer

template molecules with the soluble primers in the presence of a primer extension polymerase, a plurality of nucleotides, and a high efficiency hybridization buffer. In some embodiment, the high efficiency hybridization buffer comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

[00443] In some embodiments, in step (e), the plurality of extended forward sequencing primer strands can be removed using an enzyme or a chemical reagent. For example, the plurality of extended forward sequencing primer strands can be enzymatically degraded using a 5' to 3' double-stranded DNA exonuclease, including T7 exonuclease (e.g., from New England Biolabs, catalog # M0263S). In some embodiments, the plurality of extended forward sequencing primer strands can be removed with a temperature that favors nucleic acid denaturation.

[00444] In some embodiments, in step (e), a denaturation reagent can be used to remove the plurality of extended forward sequencing primer strands, wherein the denaturation reagent comprises any one or any combination of compounds such as formamide, acetonitrile, guanidinium chloride and/or a buffering agent (e.g., Tris-HCl, MES, HEPES, or the like).

[00445] In some embodiments, in step (e), the plurality of extended forward sequencing primer strands can be removed using an elevated temperature (e.g., heat) with or without a nucleic acid denaturation reagent. The plurality of extended forward sequencing primer strands can be subjected to a temperature of about 45-50 °C, or about 50-60 °C, or about 60-70 °C, or about 70-80 °C, or about 80-90 °C, or about 90-95 °C, or higher temperature.

[00446] In some embodiments, in step (e), the plurality of extended forward sequencing primer strands can be removed using 100% formamide at a temperature of about 65 °C for about 3 minutes, and washing with a reagent comprising about 50 mM NaCl or equivalent ionic strength and having a pH of about 6.5 – 8.5.

[00447] In some embodiments, the primer extension polymerase of step (e) comprises a high fidelity polymerase. In some embodiments, the primer extension polymerase of step (e) comprises a DNA polymerase capable of catalyzing a primer extension reaction using a uracil-containing template molecule (e.g., a uracil-tolerant polymerase). Exemplary polymerases include, but are not limited to, Q5U Hot Start high-fidelity DNA polymerase (e.g., catalog # M0515S from New England Biolabs), Taq DNA polymerase, One Taq DNA polymerase (e.g., mixture of Taq and Deep Vent DNA polymerases, catalog #M0480S from New England Biolabs), LongAmp Taq DNA polymerase (e.g., catalog #M0323S from New England Biolabs), Epimark Hot Start Taq DNA polymerase (e.g., catalog #M0490S from New England Biolabs), Bst DNA polymerase (e.g., large fragment, catalog #M0275S from New England Biolabs), Bsu DNA polymerase (e.g., large fragment, catalog #M0330S from New England Biolabs), Phi29 DNA polymerase (e.g., catalog # M0269S from New England Biolabs), *E. coli* DNA polymerase (e.g., catalog # M0209S from New England Biolabs), Therminator DNA polymerase (e.g., catalog #M0261S from New England Biolabs), Vent DNA polymerase and Deep Vent DNA polymerase.

[00448] The pairwise methods described herein can provide increased accuracy in a downstream sequencing reaction because step (e) replaces the extended forward sequencing primer strands that were generated in step (d) with forward extension strands having reduced base errors. The extended forward sequencing primer strands are generated in step (d) and may or may not contain erroneously incorporated nucleotides due to polymerase-catalyzed mis-paired bases. When step (e) is conducted with a high fidelity DNA polymerase, the resulting forward extension strands may have reduced base errors compared to the extended forward sequencing primer strands. The forward extension strands will be used as a nucleic acid template for a downstream sequencing step (e.g., see step (f) below). Thus, step (e) can increase the sequencing accuracy of the downstream step (g) and therefore increase the overall sequencing accuracy of the pairwise sequencing workflow.

[00449] In some embodiments, the pairwise sequencing method further comprises **step (f):** removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized surface primers (Figures 46 and 48).

[00450] The abasic sites are generated on the retained concatemer template strands that contain nucleotides having scissile moieties. In some embodiments, the scissile moieties in the retained concatemer template molecules comprises uridine, 8-oxo-7,8-dihydroguanine (e.g., 8oxoG) or deoxyinosine. The abasic sites can be removed to generate a plurality of single stranded nucleic acid template molecules having gaps while retaining the plurality of forward extension strands. The abasic sites can be generated by contacting the immobilized concatemer template molecules with an enzyme that removes the nucleo-base at the nucleotide having the scissile moiety. The uracil in the retained concatemer template strands can be converted to an abasic site using uracil DNA glycosylase (UDG). The 8oxoG in the retained concatemer template strands can be converted to an abasic site using FPG glycosylase. The deoxyinosine in the retained concatemer template strands can be converted to an abasic site using AlkA glycosylase.

[00451] In some embodiments, in step (f), the gaps can be generated by contacting the abasic sites in the immobilized concatemer template molecules with an enzyme or a mixture of enzymes having lyase activity that breaks the phosphodiester backbone at the 5' and 3' sides of the abasic site to release the base-free deoxyribose and generate a gap (Figures 46 and 48). The abasic sites can be removed using AP lyase, Endo IV endonuclease, FPG glycosylase/AP lyase, Endo VIII glycosylase/AP lyase. In some embodiments, generating the abasic sites and removal of the abasic sites to generate gaps can be achieved using a mixture of uracil DNA glycosylase and DNA glycosylase-lyase endonuclease VIII, for example USER (Uracil-Specific Excision Reagent Enzyme from New England Biolabs) or thermolabile USER (also from New England Biolabs).

[00452] In some embodiments, in step (f), the plurality of gap-containing template molecules can be removed using an enzyme, chemical compound and/or heat. After the gap-removal

procedure, the plurality of retained forward extension strands can be hybridized to the retained immobilized surface primers (Figures 47 and 49).

[00453] For example, the plurality of gap-containing template molecules can be enzymatically degraded using a 5' to 3' double-stranded DNA exonuclease, including T7 exonuclease (e.g., from New England Biolabs, catalog # M0263S). When a 5' to 3' double-stranded DNA exonuclease is used for removing gap-containing template molecules, then the plurality of soluble amplification primers in step (e) can comprise at least one phosphorothioate diester bond at their 5' ends which can render the soluble amplification primers resistant to exonuclease degradation. In some embodiments, the plurality of soluble amplification primers in step (e) comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5' ends. In some embodiments, the plurality soluble amplification primers in step (e) comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the forward sequencing primers resistant to exonuclease degradation.

[00454] In some embodiments, the plurality of gap-containing template molecules can be removed using a chemical reagent that favors nucleic acid denaturation. The denaturation reagent can include any one or any combination of compounds such as formamide, acetonitrile, guanidinium chloride and/or a buffering agent (e.g., Tris-HCl, MES, HEPES, or the like).

[00455] In some embodiments, the plurality of gap-containing template molecules can be removed using an elevated temperature (e.g., heat) with or without a nucleic acid denaturation reagent. The gap-containing template molecules can be subjected to a temperature of about 45-50 °C, or about 50-60 °C, or about 60-70 °C, or about 70-80 °C, or about 80-90 °C, or about 90-95 °C, or higher temperature.

[00456] In some embodiments, the plurality of gap-containing template molecules can be removed using 100% formamide at a temperature of about 65 °C for about 3 minutes, and washing with a reagent comprising about 50 mM NaCl or equivalent ionic strength and having a pH of about 6.5 – 8.5.

[00457] In some embodiments, the pairwise sequencing method further comprises **step (g):** sequencing the plurality of retained forward extension strands thereby generating a plurality of extended reverse sequencing primer strands. In some embodiments, the sequencing of step (g) comprises contacting the plurality of retained forward extension strands with a plurality of soluble reverse sequencing primers under a condition suitable to hybridize the reverse

sequencing primers to the reverse sequencing primer binding site of the retained forward extension strands, and by conducting sequencing reactions using the hybridized reverse sequencing primers wherein the forward sequencing reactions generates a plurality of extended reverse sequencing primer strands (Figures 50 and 51). The extended reverse sequencing primer strands are hybridized to the retained forward extension strand. The retained forward extension strand is hybridized to the first surface primer. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support.

[00458] For the sake of simplicity, Figures 47 and 49 show exemplary retained forward extension strands each having either (i) one copy of the sequence of interest and various universal primer binding sites (Figure 47) or (ii) two tandem copies of the sequence of interest and various universal primer binding sites (Figure 49). The skilled artisan will appreciate that the retained forward extension strand can include two, three, four or many more tandem copies containing the sequence of interest and various universal primer binding sites. Therefore, the reverse sequencing reaction can generate a plurality of extended reverse sequencing primer strands hybridized to the same retained forward extension strand.

[00459] In some embodiments, in step (g), the condition suitable to hybridize the reverse sequencing primers to the reverse sequencing primer binding sequences of the retained forward extension strands comprises contacting the plurality of soluble reverse sequencing primers and the retained forward extension strands with a high efficiency hybridization buffer. In some embodiments, the high efficiency hybridization buffer comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises

polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

[00460] In an alternative embodiment, the sequencing of step (g) comprises using the immobilized surface primer as a sequencing primer and conducting sequencing reactions to generate a plurality of reverse sequencing strands.

[00461] In some embodiments, the reverse sequencing reactions of step (g) comprises contacting the plurality of reverse sequencing primers with the reverse sequencing primer binding sequences of the retained forward extension strands, one or more types of sequencing polymerases, and a plurality of nucleotides and/or a plurality of multivalent molecules. In some embodiments, the soluble reverse sequencing primers comprise 3' OH extendible ends. In some embodiments, the soluble reverse sequencing primers comprise a 3' blocking moiety which can be removed to generate a 3' OH extendible end. In some embodiments, the soluble reverse sequencing primers lack a nucleotide having a scissile moiety. The sequencing reactions that employ nucleotides and/or multivalent molecules is described in more detail below. The reverse sequencing reactions can generate a plurality of extended reverse sequencing primer strands. In some embodiments, individual retained forward extension strands have multiple copies of the reverse sequencing primer binding sequences/sites, wherein each reverse sequencing primer binding site is capable of hybridizing to a reverse sequencing primer. Individual reverse sequencing primer binding sites in a given retained forward extension strand can be hybridized to a reverse sequencing primer and can undergo a sequencing reaction. Thus, an individual retained forward extension strand can undergo two or more sequence reactions, where each sequencing reaction is initiated from a reverse sequencing primer that is hybridized to a reverse sequencing primer binding site (Figures 50 and 51). In some embodiments, the sequencing reactions comprise a plurality of nucleotides (or analogs thereof) labeled with a detectable reporter moiety. In some embodiments, the sequencing reaction comprise a plurality of multivalent molecules having nucleotide units, where the multivalent molecules are labeled with a detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore.

[00462] In some embodiments, at least one washing step can be conducted after any of steps (a) – (g). The washing step can be conducted with a wash buffer comprising a pH buffering agent, a metal chelating agent, a salt, and a detergent.

[00463] In some embodiments, the pH buffering compound in the wash buffer comprises any one or any combination of two or more of Tris, Tris-HCl, Tricine, Bicine, Bis-Tris propane, HEPES, MES, MOPS, MOPSO, BES, TES, CAPS, TAPS, TAPSO, ACES, PIPES, ethanolamine (a.k.a 2-amino methanol; MEA), a citrate compound, a citrate mixture, NaOH and/or KOH. In some embodiments, the pH buffering agent can be present in the wash buffer at a concentration of about 1-100 mM, or about 10-50 mM, or about 10-25 mM. In some embodiments, the pH of the pH buffering agent which is present in any of the reagents described here in can be adjusted to a pH of about 4-9, or a pH of about 5-9, or a pH of about 5-8.

[00464] In some embodiments, the metal chelating agent in the wash buffer comprises EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol tetraacetic acid), HEDTA (hydroxyethylethylenediaminetriacetic acid), DPTA (diethylene triamine pentaacetic acid), NTA (N,N-bis(carboxymethyl)glycine), citrate anhydrous, sodium citrate, calcium citrate, ammonium citrate, ammonium bicitrate, citric acid, potassium citrate, or magnesium citrate. In some embodiments, the wash buffer comprises a chelating agent at a concentration of about 0.01 – 50 mM, or about 0.1 – 20 mM, or about 0.2 – 10 mM.

[00465] In some embodiments, the salt in the wash buffer comprises NaCl, KCl, NH_2SO_4 or potassium glutamate. In some embodiments, the detergent comprises an ionic detergent such as SDS (sodium dodecyl sulfate). The wash buffer can include a monovalent salt at a concentration of about 25-500 mM, or about 50-250 mM, or about 100-200 mM.

[00466] In some embodiments, the detergent in the wash buffer comprises a non-ionic detergent such as Triton X-100, Tween 20, Tween 80 or Nonidet P-40. In some embodiments, the detergent comprises a zwitterionic detergent such as CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) or *N*-Dodecyl-*N,N*-dimethyl-3-amonio-1-propanesulfate (DetX). In some embodiments, the detergent comprises LDS (lithium dodecyl sulfate), sodium taurodeoxycholate, sodium taurocholate, sodium glycocholate, sodium deoxycholate or sodium cholate. In some embodiments, the detergent is included in the wash buffer at a concentration of about 0.01-0.05%, or about 0.05-0.1%, or about 0.1-0.15%, or about 0.15-0.2%, or about 0.2-0.25%.

On Support Ligation and RCA and Pairwise Sequencing

[00467] The present disclosure provides pairwise sequencing methods, comprising **step (a):** providing a support having a plurality of surface primers (e.g., a plurality of first surface primers)

immobilized thereon, wherein individual first surface primers in the plurality comprise a first portion (SP1-A) and a second portion (SP1-B), and the individual first surface primers comprising a 3' extendible end and lacking a nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the first surface primer. In some embodiments, the immobilized first surface primers lack a nucleotide having a scissile moiety (Figure 55). For example, the surface primers lack uridine, 8-oxo-7,8-dihydroguanine (e.g., 8oxoG) and deoxyinosine. In some embodiments, the first and second portions (SP1-A and SP1-B) of the first surface primers have the same or different lengths. The first portion (SP1-A) of the first surface primers can be about 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths. The second portion (SP1-B) of the first surface primers can be about 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths. In some embodiments, the first and second portions (SP1-A and SP1-B) of the immobilized first surface primers have the same or different sequences. In some embodiments, the support comprises a plurality of first surface primers. In some embodiments, the support lacks a plurality of second surface primers. In some embodiments, the support comprises a plurality of first and second surface primers.

[00468] In some embodiments, the immobilized first surface primers comprise single stranded oligonucleotides comprising DNA, RNA or a combination of DNA and RNA. The first surface primers comprise a sequence that is wholly complementary or partially complementary along their lengths to at least a portion of a nucleic acid library molecule (e.g., linear or circular library molecules). The first surface primers can include a terminal 3' nucleotide having a sugar 3' OH moiety which is extendible for nucleotide polymerization (e.g., polymerase catalyzed polymerization).

[00469] The immobilized first surface primers can be immobilized to the support or immobilized to a coating on the support. The immobilized first surface primers can be embedded and attached (coupled) to the coating on the support. In some embodiments, the 5' end of the immobilized first surface primers are immobilized to a support or immobilized to a coating on the support. Alternatively, an interior portion or the 3' end of the immobilized first surface primers can be immobilized to a support or immobilized to a coating on the support. The support comprises a plurality of immobilized first surface primers having the same sequence. The

immobilized first surface primers can be any length, for example 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths.

[00470] In some embodiments, the plurality of immobilized first surface primers comprise at least one phosphorothioate diester bond at their 5' ends which can render the first surface primers resistant to exonuclease degradation. In some embodiments, the plurality of immobilized first surface primers comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5' ends. In some embodiments, the plurality of immobilized first surface primers comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the first surface primers resistant to exonuclease degradation.

[00471] In some embodiments, the immobilized first surface primers comprise at least one locked nucleic acid (LNA) which comprises a methylene bridge bond between a 2' oxygen and 4' carbon of the pentose ring. Immobilized first surface primers that include at least one LNA can be resistant to nuclease digestions and can exhibit increased melting temperature when hybridized to the forward extension strand.

[00472] In some embodiments, the support further comprises a plurality of a second surface primer immobilized thereon (Figure 72). The second surface primers have a sequence that differs from the first immobilized surface primer. The immobilized second surface primers of step (a) comprise single stranded oligonucleotides comprising DNA, RNA or a combination of DNA and RNA. The second surface primers comprise a sequence that is wholly complementary or partially complementary along their lengths to at least a portion of an immobilized single stranded concatemer template molecule. The immobilized second surface primers can be immobilized to the support or immobilized to a coating on the support. The immobilized second surface primers can be embedded and attached (coupled) to the coating on the support. In some embodiments, the 5' end of the immobilized second surface primers are immobilized to a support or immobilized to a coating on the support. Alternatively, an interior portion or the 3' end of the immobilized second surface primers can be immobilized to a support or immobilized to a coating on the support. The support comprises a plurality of immobilized second surface primers having the same sequence. The immobilized second surface primers can be any length, for example 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths. In some embodiments, the 3' terminal end of the immobilized second surface primers comprise an extendible 3' OH moiety. In some embodiments, the 3' terminal end of the immobilized second

surface primers comprise a 3' non-extendible moiety. The 3' terminal end of the immobilized second surface primers comprise a moiety that blocks primer extension, such as for example a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group. The immobilized second surface primers are not extendible in a primer extension reaction. The immobilized second surface primers lack a nucleotide having a scissile moiety.

[00473] In some embodiments, the plurality of immobilized second surface primers comprise at least one phosphorothioate diester bond at their 5' ends which can render the second surface primers resistant to exonuclease degradation. In some embodiments, the plurality of immobilized second surface primers comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5' ends. In some embodiments, the plurality of immobilized second surface primers comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the second surface primers resistant to exonuclease degradation.

[00474] In some embodiments, individual immobilized single stranded nucleic acid concatemer template molecule are covalently joined to an immobilized first surface primer, and at least one portion of the individual concatemer template molecule is hybridized to an immobilized second surface primer (Figure 72). The immobilized second surface primers serve to pin down a portion of the immobilized concatemer template molecules to the support. The immobilized concatemer template molecule has two or more copies of a universal binding sequence for an immobilized second surface primer. The portion of the immobilized concatemer template molecule that includes the universal binding sequence for an immobilized second surface primer can hybridize to the immobilized second surface primer. In some embodiments, the second surface primers include a terminal 3' blocking group that renders them non-extendible. In some embodiments, the second surface primers have terminal 3' extendible ends.

[00475] In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized first surface primers per mm^2. In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized second surface primers per mm^2. In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized first surface primers and immobilized second surface primers per mm^2.

[00476] The immobilized surface primers (e.g., first and second surface primers) are in fluid communication with each other to permit flowing various solutions of linear or circular nucleic acid template molecules, soluble primers, enzymes, nucleotides, divalent cations, buffers,

reagents, and the like, onto the support so that the plurality of immobilized surface primers (and the primer extension products generated from the immobilized surface primers) react with the solutions in a massively parallel manner.

[00477] In some embodiments, the pairwise sequencing method further comprises **step (b):** contacting the plurality of the first surface primers with a plurality of single stranded linear nucleic acid library molecules each library molecule having 5' and 3' ends. The contacting is conducted under a condition suitable for hybridizing individual library molecules to an immobilized first surface primer to form a circularized library molecule having a gap or nick between the 5' and 3' ends of the circularized library molecule (Figures 57 and 58).

[00478] In some embodiments, the position of the gap or nick in the circularized library molecules can be asymmetrical or symmetrical relative to the duplex formed by hybridizing the 5' and 3' ends of the linear library molecule to the immobilized first surface primers. For example, Figure 57 shows an asymmetrical positioned gap or nick. Figure 58 (left) shows an asymmetrical positioned gap or nick. Figure 58 (right) shows a symmetrical positioned gap or nick. An asymmetrical or symmetrical positioned gap/nick can be generated by adjusting the length of the first portion (SP1-A) and the second portion (SP1-B) in the immobilized first surface primers.

[00479] In some embodiments, individual library molecules in the plurality comprise a sequence of interest and the library molecules further comprise any one or any combination of two or more of: (i) a universal binding sequence (or complementary sequence thereof) for a soluble forward sequencing primer; (ii) a universal binding sequence (or complementary sequence thereof) for a soluble reverse sequencing primer; (iii) a universal binding sequence (or complementary sequence thereof) for a first portion of an immobilized first surface primer (SP1-A); (iv) a universal binding sequence (or complementary sequence thereof) for a second portion of an immobilized first surface primer (SP1-B); (v) a universal binding sequence (or complementary sequence thereof) for an immobilized second surface primer; (vi) a universal binding sequence (or complementary sequence thereof) for a first soluble amplification primer; (vii) a universal binding sequence (or complementary sequence thereof) for a second soluble amplification primer; (viii) a universal binding sequence (or complementary sequence thereof) for a soluble compaction oligonucleotide; (ix) a sample barcode sequence and/or (x) a unique

molecular index sequence. An exemplary single stranded linear library molecule is shown in Figure 56.

[00480] In some embodiments, the universal binding sequence for a first portion of an immobilized first surface primer (e.g., SP1-A') in the linear library molecule can hybridize to the first portion of the immobilized first surface primer (SP1-A). In some embodiments, the universal binding sequence for a second portion of an immobilized first surface primer (e.g., SP1-B') in the linear library molecule can hybridize to the second portion of the immobilized first surface primer (SP1-B). In some embodiments, the immobilized first surface primers comprise a first portion (SP1-A) and a second portion (SP1-B) which hybridize to SP1-A' and SP1-B' in the linear library molecule, and the first surface primers serve as a nucleic acid splint molecule for circularizing the linear library molecules.

[00481] In some embodiments, the pairwise sequencing method further comprises **step (c):** enzymatically closing the gap or nick thereby forming individual single stranded covalently closed circular molecules that are hybridized to an immobilized first surface primer (Figure 59, Figure 60 (left) and Figure 60 (right)).

[00482] In some embodiments, the gap in the circularized library molecule is closed by conducting a polymerase-catalyzed gap fill-in reaction using the 3' extendible end of the library molecule as an initiation site for the polymerase-catalyzed fill-in reaction and using the immobilized first surface primer as a template molecule thereby forming circularized molecule having a nick. The nick is closed by conducting an enzymatic ligation reaction to form a single stranded covalently closed circular molecule, wherein individual covalently closed circular molecules are hybridized to an immobilized first surface primer. In some embodiments, the gap fill-in reaction can be conducting with a plurality of nucleotides and a polymerase that lacks 5' to 3' strand displacement activity. The polymerase comprises *E. coli* DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T7 DNA polymerase, or T4 DNA polymerase. In some embodiments, the ligation reaction can be conducted using a DNA ligase which comprises a T3, T4, T7 or Taq DNA ligase.

[00483] In some embodiments, the nick in the circularized library molecule is closed by conducting a ligase-catalyzed ligation reaction to form a single stranded covalently closed circular molecule, wherein individual covalently closed circular molecules are hybridized to an

immobilized first surface primer. In some embodiments, the ligase enzyme comprises T3, T4, T7 or Taq DNA ligase.

[00484] In some embodiments, the pairwise sequencing method further comprises **step (d):** generating a plurality of immobilized single stranded nucleic acid concatemer template molecules by conducting a rolling circle amplification reaction with a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety that can be cleaved to generate an abasic site, thereby generating a plurality of immobilized single stranded nucleic acid concatemer template molecules having at least one nucleotide with a scissile moiety, wherein individual single stranded nucleic acid concatemer template molecules are covalently joined to an immobilized first surface primer (Figure 61). In some embodiments, the rolling circle amplification reaction can be conducted in the presence, or in the absence, of a plurality of a plurality of compaction oligonucleotides.

[00485] In some embodiments, the single-stranded circular nucleic acid library molecules can be removed from the concatemer template molecules with at least one washing step which is conducted under a condition suitable to retain the single stranded nucleic acid concatemer template molecules where individual concatemer template molecules are operably joined to an immobilized first surface primer.

[00486] In some embodiments, individual immobilized concatemer template molecules generated by the rolling circle amplification reaction comprise two or more copies of a sequence of interest and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of: (i) two or more copies of a universal binding sequence for a soluble forward sequencing primer; (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer; (iii) two or more copies of a universal binding sequence for a first portion of an immobilized first surface primer (SP1-A); (iv) two or more copies of a universal binding sequence for a second portion of an immobilized first surface primer (SP1-B); (v) two or more copies of a universal binding sequence for an immobilized second surface primer; (vi) two or more copies of a universal binding sequence for a first soluble amplification primer (vii) two or more copies of a universal binding sequence for a second soluble amplification primer; (viii) two or more copies of a universal binding sequence for a

soluble compaction oligonucleotide; (ix) two or more copies of a sample barcode sequence and/or (x) two or more copies of a unique molecular index sequence.

[00487] In some embodiments, the plurality of immobilized single stranded nucleic acid concatemer template molecules that are generated by the rolling circle amplification reaction of step (d) further comprise two or more copies of a universal binding sequence (or complementary sequence thereof) for immobilized second sequence surface primers. In some embodiments, individual immobilized single stranded nucleic acid concatemer template molecule are joined (e.g., covalently joined) to an immobilized first surface primer, and at least one portion of the individual concatemer template molecule is hybridized to an immobilized second surface primer. The immobilized second surface primers serve to pin down a portion of the immobilized concatemer template molecules to the support (see Figure 72). In some embodiments, the second surface primers include a terminal 3' blocking group that renders them non-extendible.

[00488] The rolling circle amplification reaction of step (d) can be conducted with a nucleotide mixture containing dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety to generate immobilized concatemer template molecules which includes at least one nucleotide having a scissile moiety. The scissile moieties in the immobilized concatemer template molecules can be converted into abasic sites. In some embodiments, in the nucleotide mixture, the nucleotide having the scissile moiety comprises uridine, 8-oxo-7,8-dihydroguanine (e.g., 8oxoG) or deoxyinosine. In the immobilized concatemer template molecules, the uridine can be converted to an abasic site using uracil DNA glycosylase (UDG), the 8oxoG can be converted to an abasic site using FPG glycosylase, and the deoxyinosine can be converted to an abasic site using AlkA glycosylase.

[00489] In some embodiments, the nucleotide mixture can include an amount of dUTP so that a target percent of the thymidine in the resulting concatemer molecules are replaced with dUTP. For example, when 30% of dTTP in the concatemer molecules are to be replaced with dUTP (e.g., 30% is the target percent) then the nucleotide mixture can contain 7.5% dUTP (e.g., 30/4 = 7.5%), 17.5% dTTP, and 25% each for dATP, dCTP and dGTP. The target percent of dTTP to be replaced by dUTP can be about 0.1-1%, or about 1-5%, or about 5-10%, or about 10-20%, or about 20-30% , or about 30-45%, or about 45-50%, or a higher percent of the dTTP in the immobilized concatemer template molecules are replaced with nucleotides having a scissile moiety.

[00490] In some embodiments, the nucleotide mixture can include an amount of deoxyinosine so that a target percent of the guanosine in the resulting concatemer molecules are replaced with deoxyinosine. For example, when 30% of dGTP in the concatemer molecules are to be replaced with deoxyinosine (e.g., 30% is the target percent) then the nucleotide mixture can contain 7.5% deoxyinosine (e.g., 30/4 = 7.5%), 17.5% dGTP, and 25% each for dATP, dCTP and dTTP. The target percent of dGTP to be replaced by deoxyinosine can be about 0.1-1%, or about 1-5%, or about 5-10%, or about 10-20%, or about 20-30% , or about 30-45%, or about 45-50%, or a higher percent of the dGTP in the immobilized concatemer template molecules are replaced with nucleotides having a scissile moiety.

[00491] In some embodiments, the nucleotide mixture can include an amount of 8oxoG so that a target percent of the guanosine in the resulting concatemer molecules are replaced with 8oxoG. For example, when 30% of dGTP in the concatemer molecules are to be replaced with 8oxoG (e.g., 30% is the target percent) then the nucleotide mixture can contain 7.5% 8oxoG (e.g., 30/4 = 7.5%), 17.5% dGTP, and 25% each for dATP, dCTP and dTTP. The target percent of dGTP to be replaced by 8oxoG can be about 0.1-1%, or about 1-5%, or about 5-10%, or about 10-20%, or about 20-30% , or about 30-45%, or about 45-50%, or a higher percent of the dGTP in the immobilized concatemer template molecules are replaced with nucleotides having a scissile moiety.

[00492] In some embodiments, the rolling circle amplification reaction generates immobilized concatemer template molecules with incorporated nucleotides having a scissile moiety that are distributed at random positions along individual immobilized concatemer template molecules. In some embodiments, the nucleotides having a scissile moiety are distributed at different positions in the different immobilized concatemer template molecules.

[00493] In some embodiments, the pairwise sequencing method further comprises **step (e):** sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands. The sequencing of step (e) comprises contacting the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers under a condition suitable to hybridize at least one forward sequencing primer to at least one of the forward sequencing primer binding sites/sequences of the immobilized concatemer template molecules, and conducting forward sequencing reactions using one or more types of sequencing polymerases, a plurality of nucleotides and/or multivalent

molecules, and the hybridized first forward sequencing primers (Figure 62). In some embodiments, the soluble forward sequencing primers comprise 3' OH extendible ends. In some embodiments, the soluble forward sequencing primers comprise a 3' blocking moiety which can be removed to generate a 3' OH extendible end. In some embodiments, the soluble forward sequencing primers lack a nucleotide having a scissile moiety. The forward sequencing reactions can generate a plurality of extended forward sequencing primer strands. In some embodiments, individual immobilized concatemer template molecules have multiple copies of the forward sequencing primer binding sites, wherein each forward sequencing primer binding site is capable of hybridizing to a first forward sequencing primer. Individual forward sequencing primer binding sites in a given immobilized concatemer template molecule can be hybridized to a forward sequencing primer and can undergo a sequencing reaction. Individual immobilized concatemer template molecules can undergo two or more sequence reactions, where each sequencing reaction is initiated from a first forward sequencing primer that is hybridized to a forward sequencing primer binding site (e.g., see Figure 62). In some embodiments, the sequencing reactions comprise a plurality of nucleotides (or analogs thereof) labeled with a detectable reporter moiety. In some embodiments, the sequencing reaction comprise a plurality of multivalent molecules having a plurality of nucleotide units attached to a core, where the multivalent molecules are labeled with a detectable reporter moiety. In some embodiments, the core is labeled with a detectable reporter moiety. In some embodiments, at least one linker and/or at least one nucleotide unit of a nucleotide arm is labeled with a detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore. An exemplary nucleotide arm is shown in Figure 108, and exemplary multivalent molecules are shown in Figures 104-107.

[00494] In some embodiments, the pairwise sequencing method further comprises **step (f):** retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules. The plurality of extended forward sequencing primer strands can be removed and replaced with a plurality of forward extension strands by conducting a primer extension reaction (see Figures 63- 65).

[00495] In some embodiments, step (f) comprises contacting at least one extended forward sequencing primer strand with a plurality of strand displacing polymerases and a plurality of nucleotides and in the absence of soluble amplification primers, under a condition suitable to conduct a strand displacing primer extension reaction using the at least one extended forward sequencing primers strand to initiate the primer extension reaction thereby generating a forward extension strand that is covalently joined to the extended forward sequencing primers strand, wherein the forward extension strand is hybridized to the immobilized concatemer template molecule (Figure 63). For example, one of the extended forward sequencing primer strands can serve as a primer for the strand displacing polymerase. The strand displacing polymerase can extend the extended forward sequencing primer strand, and displace downstream extended forward sequencing primer strands while synthesizing an extended strand that replaces the downstream extended forward sequencing primer strands. The newly extended strand is covalently joined to an extended forward sequencing primer strand. The immobilized concatemer template molecules are retained. The primer extension reaction can optionally include a plurality of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) to generate forward extension strands. Individual forward extension strands can collapse into a nanoball having a more compact size and/or shape compared to a nanoball generated from a primer extension reaction conducted without compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III). Inclusion of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) in the primer extension reaction can improve FWHM (full width half maximum) of a spot image of the nanoball. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

[00496] Examples of strand displacing polymerases include phi29 DNA polymerase, large fragment of Bst DNA polymerase, large fragment of Bsu DNA polymerase (exo-), Bca DNA polymerase (exo-), Klenow fragment of E. coli DNA polymerase, T5 polymerase, M-MuLV reverse transcriptase, HIV viral reverse transcriptase, Deep Vent DNA polymerase and KOD DNA polymerase. The phi29 DNA polymerase can be wild type phi29 DNA polymerase (e.g., MagniPhi from Expedeon), or variant EquiPhi29 DNA polymerase (e.g., from Thermo Fisher Scientific), or chimeric QualiPhi DNA polymerase (e.g., from 4basebio).

[00497] In some embodiments, step (f) comprises: **(i)** removing the plurality of extended forward sequencing primer strand while retaining the immobilized concatemer template molecules; and **(ii)** contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble forward sequencing primers (e.g., a second plurality of soluble forward sequencing primers), a plurality of nucleotides (e.g., a second plurality of nucleotides) and a plurality of primer extension polymerases, under a condition suitable to hybridize the plurality of soluble forward sequencing primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed primer extension reactions thereby generating a plurality of forward extension strands, wherein the soluble sequencing primers hybridize with the forward sequencing primer binding sequence in the retained immobilized concatemer molecules (Figure 64). The primer extension reaction can optionally include a plurality of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) to generate forward extension strands. Individual forward extension strands can collapse into a nanoball having a more compact size and/or shape compared to a nanoball generated from a primer extension reaction conducted without compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III). Inclusion of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) in the primer extension reaction can improve FWHM (full width half maximum) of a spot image of the nanoball. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

[00498] In some embodiments, in step (f), the condition suitable to hybridize the plurality of soluble forward sequencing primers to the plurality of retained immobilized single stranded nucleic acid concatemer template molecules comprises hybridizing retained immobilized concatemer template molecules with the soluble primers in the presence of a primer extension polymerase, a plurality of nucleotides, and a high efficiency hybridization buffer. In some embodiment, the high efficiency hybridization buffer comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer

formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

[00499] In some embodiments, step (f) comprises: **(i)** removing the plurality of extended forward sequencing primer strand while retaining the immobilized concatemer template molecules; and **(ii)** contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble amplification primers, a plurality of nucleotides (e.g., a second plurality of nucleotides) and a plurality of primer extension polymerases, under a condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed primer extension reactions thereby generating a plurality of forward extension strands, wherein the soluble amplification primers hybridize with the soluble amplification primer binding sequence in the retained immobilized concatemer molecules (Figure 65). The primer extension reaction can optionally include a plurality of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) to generate forward extension strands. Individual forward extension strands can collapse into a nanoball having a more compact size and/or shape compared to a nanoball generated from a primer extension reaction conducted without compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III). Inclusion of compaction oligonucleotides and/or hexamine (e.g., cobalt hexamine III) in the primer extension reaction can improve FWHM (full width half maximum) of a spot image of the nanoball. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

[00500] In some embodiments, in step (f), the condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized single stranded nucleic acid concatemer template molecules comprises hybridizing retained immobilized concatemer

template molecules with the soluble primers in the presence of a primer extension polymerase, a plurality of nucleotides, and a high efficiency hybridization buffer. In some embodiment, the high efficiency hybridization buffer comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

[00501] In some embodiments, in step (f), the plurality of extended forward sequencing primer strands can be removed using an enzyme or a chemical reagent. For example, the plurality of extended forward sequencing primer strands can be enzymatically degraded using a 5' to 3' double-stranded DNA exonuclease, including T7 exonuclease (e.g., from New England Biolabs, catalog # M0263S). In some embodiments, the plurality of extended forward sequencing primer strands can be removed with a temperature that favors nucleic acid denaturation.

[00502] In some embodiments, in step (f), a denaturation reagent can be used to remove the plurality of extended forward sequencing primer strands, wherein the denaturation reagent comprises any one or any combination of compounds such as formamide, acetonitrile, guanidinium chloride and/or a buffering agent (e.g., Tris-HCl, MES, HEPES, or the like).

[00503] In some embodiments, in step (f), the plurality of extended forward sequencing primer strands can be removed using an elevated temperature (e.g., heat) with or without a nucleic acid denaturation reagent. The plurality of extended forward sequencing primer strands can be subjected to a temperature of about 45-50 °C, or about 50-60 °C, or about 60-70 °C, or about 70-80 °C, or about 80-90 °C, or about 90-95 °C, or higher temperature.

[00504] In some embodiments, in step (f), the plurality of extended forward sequencing primer strands can be removed using 100% formamide at a temperature of about 65 °C for about 3 minutes, and washing with a reagent comprising about 50 mM NaCl or equivalent ionic strength and having a pH of about 6.5 – 8.5.

[00505] In some embodiments, the primer extension polymerase of step (f) comprises a high fidelity polymerase. In some embodiments, the primer extension polymerase of step (d) comprises a DNA polymerase capable of catalyzing a primer extension reaction using a uracil-containing template molecule (e.g., a uracil-tolerant polymerase). Exemplary polymerases include, but are not limited to, Q5U Hot Start high-fidelity DNA polymerase (e.g., catalog # M0515S from New England Biolabs), Taq DNA polymerase, One Taq DNA polymerase (e.g., mixture of Taq and Deep Vent DNA polymerases, catalog #M0480S from New England Biolabs), LongAmp Taq DNA polymerase (e.g., catalog #M0323S from New England Biolabs), Epimark Hot Start Taq DNA polymerase (e.g., catalog #M0490S from New England Biolabs), Bst DNA polymerase (e.g., large fragment, catalog #M0275S from New England Biolabs), Bsu DNA polymerase (e.g., large fragment, catalog #M0330S from New England Biolabs), Phi29 DNA polymerase (e.g., catalog # M0269S from New England Biolabs), *E. coli* DNA polymerase (e.g., catalog # M0209S from New England Biolabs), Therminator DNA polymerase (e.g., catalog #M0261S from New England Biolabs), Vent DNA polymerase and Deep Vent DNA polymerase.

[00506] The pairwise methods described herein can provide increased accuracy in a downstream sequencing reaction because step (f) replaces the extended forward sequencing primer strands that were generated in step (e) with forward extension strands having reduced base errors. The extended forward sequencing primer strands are generated in step (e) and may or may not contain erroneously incorporated nucleotides due to polymerase-catalyzed mis-paired bases. When step (e) is conducted with a high fidelity DNA polymerase, the resulting forward extension strands may have reduced base errors compared to the extended forward sequencing primer strands. The forward extension strands will be used as a nucleic acid template for a downstream sequencing step (e.g., see step (h) below). Thus, step (f) can increase the sequencing accuracy of the downstream step (h) and therefore increase the overall sequencing accuracy of the pairwise sequencing workflow.

[00507] In some embodiments, the pairwise sequencing method further comprises **step (g):** removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized surface primers (Figures 66 and 67, and Figures 68 and 69).

[00508] The abasic sites are generated on the retained concatemer template strands that contain nucleotides having scissile moieties. In some embodiments, the scissile moieties in the retained concatemer template molecules comprises uridine, 8-oxo-7,8-dihydroguanine (e.g., 8oxoG) or deoxyinosine. The abasic sites can be removed to generate a plurality of single stranded nucleic acid template molecules having gaps while retaining the plurality of forward extension strands. The abasic sites can be generated by contacting the immobilized concatemer template molecules with an enzyme that removes the nucleo-base at the nucleotide having the scissile moiety. The uracil in the retained concatemer template strands can be converted to an abasic site using uracil DNA glycosylase (UDG). The 8oxoG in the retained concatemer template strands can be converted to an abasic site using FPG glycosylase. The deoxyinosine in the retained concatemer template strands can be converted to an abasic site using AlkA glycosylase.

[00509] In some embodiments, in step (g), the gaps can be generated by contacting the abasic sites in the immobilized concatemer template molecules with an enzyme or a mixture of enzymes having lyase activity that breaks the phosphodiester backbone at the 5' and 3' sides of the abasic site to release the base-free deoxyribose and generate a gap (Figures 66 and 68). The abasic sites can be removed using AP lyase, Endo IV endonuclease, FPG glycosylase/AP lyase, Endo VIII glycosylase/AP lyase. In some embodiments, generating the abasic sites and removal of the abasic sites to generate gaps can be achieved using a mixture of uracil DNA glycosylase and DNA glycosylase-lyase endonuclease VIII, for example USER (Uracil-Specific Excision Reagent Enzyme from New England Biolabs) or thermolabile USER (also from New England Biolabs).

[00510] In some embodiments, in step (g), the plurality of gap-containing template molecules can be removed using an enzyme, chemical compound and/or heat. After the gap-removal

procedure, the plurality of retained forward extension strands are hybridized to the retained immobilized surface primers (Figures 67 and 69).

[00511] For example, the plurality of gap-containing template molecules can be enzymatically degraded using a 5' to 3' double-stranded DNA exonuclease, including T7 exonuclease (e.g., from New England Biolabs, catalog # M0263S). When a 5' to 3' double-stranded DNA exonuclease is used for removing gap-containing template molecules, then the plurality of soluble amplification primers in step (f) can comprise at least one phosphorothioate diester bond at their 5' ends which can render the soluble amplification primers resistant to exonuclease degradation. In some embodiments, the plurality of soluble amplification primers in step (f) comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5' ends. In some embodiments, the plurality soluble amplification primers in step (f) comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the forward sequencing primers resistant to exonuclease degradation.

[00512] In some embodiments, the plurality of gap-containing template molecules can be removed using a chemical reagent that favors nucleic acid denaturation. The denaturation reagent can include any one or any combination of compounds such as formamide, acetonitrile, guanidinium chloride and/or a buffering agent (e.g., Tris-HCl, MES, HEPES, or the like).

[00513] In some embodiments, the plurality of gap-containing template molecules can be removed using an elevated temperature (e.g., heat) with or without a nucleic acid denaturation reagent. The gap-containing template molecules can be subjected to a temperature of about 45-50 °C, or about 50-60 °C, or about 60-70 °C, or about 70-80 °C, or about 80-90 °C, or about 90-95 °C, or higher temperature.

[00514] In some embodiments, the plurality of gap-containing template molecules can be removed using 100% formamide at a temperature of about 65 °C for about 3 minutes, and washing with a reagent comprising about 50 mM NaCl or equivalent ionic strength and having a pH of about 6.5 – 8.5.

[00515] In some embodiments, the pairwise sequencing method further comprises **step (h):** sequencing the plurality of retained forward extension strands thereby generating a plurality of extended reverse sequencing primer strands. In some embodiments, the sequencing of step (h) comprises contacting the plurality of retained forward extension strands with a plurality of soluble reverse sequencing primers under a condition suitable to hybridize the reverse

sequencing primers to the reverse sequencing primer binding site of the retained forward extension strands, and by conducting sequencing reactions using the hybridized reverse sequencing primers wherein the forward sequencing reactions generates a plurality of extended reverse sequencing primer strands (Figures 70 and 71). The extended reverse sequencing primer strands are hybridized to the retained forward extension strand. The retained forward extension strand is hybridized to the first surface primer. The extended reverse sequencing primer strands are not hybridized to the first surface primer, or covalently joined to the first surface primer. Therefore, the extended reverse sequencing primer strands are not immobilized to the support.

[00516] In some embodiments, in step (h), the condition suitable to hybridize the reverse sequencing primers to the reverse sequencing primer binding sequences of the retained forward extension strands comprises contacting the plurality of soluble reverse sequencing primers and the retained forward extension strands with a high efficiency hybridization buffer. In some embodiments, the high efficiency hybridization buffer comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

[00517] In an alternative embodiment, the sequencing of step (h) comprises using the immobilized surface primer as a sequencing primer and conducting sequencing reactions to generate a plurality of reverse sequencing strands.

[00518] In some embodiments, the reverse sequencing reactions of step (h) comprises contacting the plurality of reverse sequencing primers with the reverse sequencing primer binding sequences of the retained forward extension strands, one or more types of sequencing

polymerases, and a plurality of nucleotides and/or a plurality of multivalent molecules. In some embodiments, the soluble reverse sequencing primers comprise 3' OH extendible ends. In some embodiments, the soluble reverse sequencing primers comprise a 3' blocking moiety which can be removed to generate a 3' OH extendible end. In some embodiments, the soluble reverse sequencing primers lack a nucleotide having a scissile moiety. The sequencing reactions that employ nucleotides and/or multivalent molecules is described in more detail below. The reverse sequencing reactions can generate a plurality of extended reverse sequencing primer strands. In some embodiments, individual retained forward extension strands have multiple copies of the reverse sequencing primer binding sequences/sites, wherein each reverse sequencing primer binding site is capable of hybridizing to a reverse sequencing primer. Individual reverse sequencing primer binding sites in a given retained forward extension strand can be hybridized to a reverse sequencing primer and can undergo a sequencing reaction. Thus, an individual retained forward extension strand can undergo two or more sequence reactions, where each sequencing reaction is initiated from a reverse sequencing primer that is hybridized to a reverse sequencing primer binding site (e.g., see Figures 70 and 71). In some embodiments, the sequencing reactions comprise a plurality of nucleotides (or analogs thereof) labeled with a detectable reporter moiety. In some embodiments, the sequencing reaction comprise a plurality of multivalent molecules having nucleotide units, where the multivalent molecules are labeled with a detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore.

[00519] In some embodiments, at least one washing step can be conducted after any of steps (a) – (h). The washing step can be conducted with a wash buffer comprising a pH buffering agent, a metal chelating agent, a salt, and a detergent.

[00520] In some embodiments, the pH buffering compound in the wash buffer comprises any one or any combination of two or more of Tris, Tris-HCl, Tricine, Bicine, Bis-Tris propane, HEPES, MES, MOPS, MOPSO, BES, TES, CAPS, TAPS, TAPSO, ACES, PIPES, ethanolamine (a.k.a 2-amino methanol; MEA), a citrate compound, a citrate mixture, NaOH and/or KOH. In some embodiments, the pH buffering agent can be present in the wash buffer at a concentration of about 1-100 mM, or about 10-50 mM, or about 10-25 mM. In some embodiments, the pH of the pH buffering agent which is present in any of the reagents described here in can be adjusted to a pH of about 4-9, or a pH of about 5-9, or a pH of about 5-8.

[00521] In some embodiments, the metal chelating agent in the wash buffer comprises EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol tetraacetic acid), HEDTA (hydroxyethylethylenediaminetriacetic acid), DPTA (diethylene triamine pentaacetic acid), NTA (N,N-bis(carboxymethyl)glycine), citrate anhydrous, sodium citrate, calcium citrate, ammonium citrate, ammonium bicitrate, citric acid, potassium citrate, or magnesium citrate. In some embodiments, the wash buffer comprises a chelating agent at a concentration of about 0.01 – 50 mM, or about 0.1 – 20 mM, or about 0.2 – 10 mM.

[00522] In some embodiments, the salt in the wash buffer comprises NaCl, KCl, NH_2SO_4 or potassium glutamate. In some embodiments, the detergent comprises an ionic detergent such as SDS (sodium dodecyl sulfate). The wash buffer can include a monovalent salt at a concentration of about 25-500 mM, or about 50-250 mM, or about 100-200 mM.

[00523] In some embodiments, the detergent in the wash buffer comprises a non-ionic detergent such as Triton X-100, Tween 20, Tween 80 or Nonidet P-40. In some embodiments, the detergent comprises a zwitterionic detergent such as CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) or *N*-Dodecyl-*N,N*-dimethyl-3-amonio-1-propanesulfate (DetX). In some embodiments, the detergent comprises LDS (lithium dodecyl sulfate), sodium taurodeoxycholate, sodium taurocholate, sodium glycocholate, sodium deoxycholate or sodium cholate. In some embodiments, the detergent is included in the wash buffer at a concentration of about 0.01-0.05%, or about 0.05-0.1%, or about 0.1-0.15%, or about 0.15-0.2%, or about 0.2-0.25%.

Methods for Pairwise Sequencing – Lacking Abasic Sites

[00524] The present disclosure provides pairwise sequencing methods, comprising **step (a):** providing a plurality of immobilized single stranded nucleic acid concatemer template molecules each lacking a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein individual concatemer template molecules in the plurality are immobilized to a first surface primer that is immobilized to a support, and wherein the immobilized first surface primer lacks a nucleotide having a scissile moiety. In some embodiments, the support comprises a plurality of first surface primers. In some embodiments, the support lacks a plurality of second surface primers. In some embodiments, the support comprises a plurality of first and second surface primers. Exemplary nucleotides having a scissile moiety include uridine, 8-oxo-7,8-dihydroguanine (e.g., 8oxoG) and deoxyinosine.

[00525] In some embodiments, individual immobilized concatemer template molecules are covalently joined to an immobilized surface primer (e.g., an immobilized first surface primer) (Figure 73). In an alternative embodiment, individual immobilized concatemer template molecules are hybridized to an immobilized surface primer (e.g., an immobilized first surface primer) (Figure 80).

[00526] In some embodiments, individual concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of: (i) two or more copies of a universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, (viii) two or more copies of a sample barcode sequence and/or (ix) two or more copies of a unique molecular index sequence.

[00527] In some embodiments, individual concatemer template molecules in the plurality comprise two or more copies of a sequence of interest and two or more copies of a universal binding sequence for a soluble compaction oligonucleotide, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of: (i) two or more copies of a universal binding sequence for a soluble forward sequencing primer, (ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer, (iii) two or more copies of a universal binding sequence for an immobilized first surface primer, (iv) two or more copies of a universal binding sequence for an immobilized second surface primer, (v) two or more copies of a universal binding sequence for a first soluble amplification primer, (vi) two or more copies of a universal binding sequence for a second soluble amplification primer, (vii) two or more copies of a sample barcode sequence and/or (viii) two or more copies of a unique molecular index sequence.

[00528] In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the forward sequencing primer can hybridize to at least a portion of the forward

sequencing primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the reverse sequencing primer can hybridize to at least a portion of the reverse sequencing primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the immobilized first surface primer can hybridize to at least a portion of the immobilized first surface primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the immobilized second surface primer can hybridize to at least a portion of the immobilized second surface primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the first soluble amplification primer can hybridize to at least a portion of the first soluble amplification primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the second soluble amplification primer can hybridize to at least a portion of the second soluble amplification primer. In some embodiments, the universal binding sequence (or a complementary sequence thereof) for the soluble compaction oligonucleotide can hybridize to at least a portion of the soluble compaction oligonucleotide.

[00529] In some embodiments, the immobilized first surface primers comprise single stranded oligonucleotides comprising DNA, RNA or a combination of DNA and RNA. The immobilized first surface primers can be immobilized to the support or immobilized to a coating on the support. The immobilized first surface primers can be embedded and attached (coupled) to the coating on the support. In some embodiments, the 5' end of the immobilized first surface primers are immobilized to a support or immobilized to a coating on the support. Alternatively, an interior portion or the 3' end of the immobilized first surface primers can be immobilized to a support or immobilized to a coating on the support. The support comprises a plurality of immobilized first surface primers having the same sequence. The immobilized first surface primers can be any length, for example 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths. In some embodiments, the 3' terminal end of the immobilized first surface primers comprise an extendible 3' OH moiety. In some embodiments, the 3' terminal end of the immobilized first surface primers comprise a 3' non-extendible moiety.

[00530] In some embodiments, the plurality of immobilized first surface primers comprise at least one phosphorothioate diester bond at their 5' ends which can render the first surface primers resistant to exonuclease degradation. In some embodiments, the plurality of immobilized first surface primers comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5'

ends. In some embodiments, the plurality of immobilized first surface primers comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the first surface primers resistant to exonuclease degradation.

[00531] In some embodiments, the immobilized first surface primers comprise at least one locked nucleic acid (LNA) which comprises a methylene bridge bond between a 2' oxygen and 4' carbon of the pentose ring. Immobilized first surface primers that include at least one LNA can be resistant to nuclease digestions and can exhibit increased melting temperature when hybridized to the forward extension strand.

[00532] In some embodiments, the immobilized concatemer template molecules further comprise two or more copies of a universal binding sequence (or complementary sequence thereof) for an immobilized second surface primer having a sequence that differs from the first immobilized surface primer. The immobilized second surface primers of step (a) comprise single stranded oligonucleotides comprising DNA, RNA or a combination of DNA and RNA. The immobilized second surface primers can be immobilized to the support or immobilized to a coating on the support. The immobilized second surface primers can be embedded and attached (coupled) to the coating on the support. In some embodiments, the 5' end of the immobilized second surface primers are immobilized to a support or immobilized to a coating on the support. Alternatively, an interior portion or the 3' end of the immobilized second surface primers can be immobilized to a support or immobilized to a coating on the support. The support comprises a plurality of immobilized second surface primers having the same sequence. The immobilized second surface primers can be any length, for example 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths.

[00533] In some embodiments, the 3' terminal end of the immobilized second surface primers comprise an extendible 3' OH moiety. In some embodiments, the 3' terminal end of the immobilized second surface primers comprise a 3' non-extendible moiety. In some embodiments, the 3' terminal end of the immobilized second surface primers comprise a moiety that blocks primer extension (e.g., non-extendible terminal 3' end), such as for example a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group. The immobilized second surface primers are not extendible in a primer extension reaction. The immobilized second surface primers lack a nucleotide having a scissile moiety.

[00534] In some embodiments, the plurality of immobilized second surface primers comprise at least one phosphorothioate diester bond at their 5' ends which can render the second surface primers resistant to exonuclease degradation. In some embodiments, the plurality of immobilized second surface primers comprise 2-5 or more consecutive phosphorothioate diester bonds at their 5' ends. In some embodiments, the plurality of immobilized second surface primers comprise at least one ribonucleotide and/or at least one 2'-O-methyl or 2'-O-methoxyethyl (MOE) nucleotide which can render the second surface primers resistant to exonuclease degradation.

[00535] In some embodiments, individual immobilized single stranded nucleic acid concatemer template molecules are joined or immobilized to an immobilized first surface primer, and at least one portion of the individual concatemer template molecule is hybridized to an immobilized second surface primer. The immobilized second surface primers serve to pin down a portion of the immobilized concatemer template molecules to the support (see Figures 79 and 86).

[00536] In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized first surface primers per mm^2. In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized second surface primers per mm^2. In some embodiments, the support comprises about $10^2 - 10^{15}$ immobilized first surface primers and immobilized second surface primers per mm^2.

[00537] The immobilized surface primers (e.g., first and second surface primers) are in fluid communication with each other to permit flowing various solutions of linear or circular nucleic acid template molecules, soluble primers, enzymes, nucleotides, divalent cations, buffers, reagents, and the like, onto the support so that the plurality of immobilized surface primers (and the primer extension products generated from the immobilized surface primers) react with the solutions in a massively parallel manner.

[00538] In some embodiments, the pairwise sequencing method further comprises **step (b):** sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands. The sequencing of step (b) comprises contacting the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers under a condition suitable to hybridize at least one forward sequencing primer to at least one of the forward sequencing primer binding sites/sequences of the immobilized concatemer template molecules, and conducting forward sequencing reactions

using one or more types of sequencing polymerases, a plurality of nucleotides and/or multivalent molecules, and the hybridized first forward sequencing primers. In some embodiments, the soluble forward sequencing primers comprise 3' OH extendible ends. In some embodiments, the soluble forward sequencing primers comprise a 3' blocking moiety which can be removed to generate a 3' OH extendible end. In some embodiments, the soluble forward sequencing primers lack a nucleotide having a scissile moiety. The forward sequencing reactions can generate a plurality of extended forward sequencing primer strands. In some embodiments, individual immobilized concatemer template molecules have multiple copies of the forward sequencing primer binding sites, wherein each forward sequencing primer binding site is capable of hybridizing to a first forward sequencing primer. Individual forward sequencing primer binding sites in a given immobilized concatemer template molecule can be hybridized to a forward sequencing primer and can undergo a sequencing reaction. Individual immobilized concatemer template molecules can undergo two or more sequence reactions, where each sequencing reaction is initiated from a first forward sequencing primer that is hybridized to a forward sequencing primer binding site (e.g., see Figures 74 and 81). In some embodiments, the sequencing reactions comprise a plurality of nucleotides (or analogs thereof) labeled with a detectable reporter moiety. In some embodiments, the sequencing reaction comprise a plurality of multivalent molecules having a plurality of nucleotide units attached to a core, where the multivalent molecules are labeled with a detectable reporter moiety. In some embodiments, the core is labeled with a detectable reporter moiety. In some embodiments, at least one linker and/or at least one nucleotide unit of a nucleotide arm is labeled with a detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore. An exemplary nucleotide arm is shown in Figure 108, and exemplary multivalent molecules are shown in Figures 104-107.

[00539] In some embodiments, the pairwise sequencing method further comprises **step (c):** retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands by conducting a primer extension reaction. The extended forward sequencing primer strands can be removed from the retained immobilized concatemer template molecules. The retained immobilized concatemer template molecule can be hybridized to a plurality of soluble amplification or sequencing primers and subjected to a primer extension reaction. The primer

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What is claimed:

1. A method for pairwise sequencing, comprising:
    a) providing a plurality of immobilized single stranded nucleic acid concatemer template molecules each comprising at least one nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein individual concatemer template molecules in the plurality are immobilized to a first surface primer that is immobilized to a support, and wherein the immobilized first surface primer lacks a nucleotide having a scissile moiety;
    b) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;
    c) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction;
    d) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized surface primers; and
    e) sequencing the plurality of retained forward extension strands thereby generating a plurality of extended reverse sequencing primer strands, wherein individual retained forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

2. The method of claim 1, wherein individual concatemer template molecules in the plurality are covalently joined to an immobilized first surface primer.

3. The method of claim 1, wherein individual concatemer template molecules in the plurality are hybridized to an immobilized first surface primer.

4. The method of claim 1, wherein individual immobilized concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of

(i) two or more copies of a universal binding sequence for a soluble forward sequencing primer,

(ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer,

(iii) two or more copies of a universal binding sequence for an immobilized first surface primer,

(iv) two or more copies of a universal binding sequence for an immobilized second surface primer,

(v) two or more copies of a universal binding sequence for a first soluble amplification primer,

(vi) two or more copies of a universal binding sequence for a second soluble amplification primer,

(vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide,

(viii) two or more copies of a sample barcode sequence and/or

(ix) two or more copies of a unique molecular index sequence.

5. The method of claim 1, wherein the sequencing of step (b) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

6. The method of claim 1, wherein the sequencing of step (e) comprises hybridizing a plurality of soluble reverse sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

7. The method of claim 4, wherein the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety.

8. The method of claim 7, wherein at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer.

9. The method of claim 7, wherein the plurality of immobilized second surface primers have 3' OH extendible ends.

10. The method of claim 7, wherein the plurality of immobilized second surface primers have 3' non-extendible ends.

11. The method of claim 10, wherein the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

12. A method for pairwise sequencing, comprising:
    a) providing a support having a plurality of a first surface primer immobilized thereon wherein each of the first surface primers have a 3' extendible end and lack a nucleotide having a scissile moiety;
    b) generating a plurality of immobilized single stranded nucleic acid concatemer template molecules by hybridizing a plurality of single-stranded circular nucleic acid library molecules to the plurality of immobilized first surface primers and conducting a rolling circle amplification reaction with a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety that can be cleaved to generate an abasic site, thereby generating a plurality of immobilized single stranded nucleic acid concatemer template molecules having at least one

nucleotide with a scissile moiety, wherein individual single stranded nucleic acid concatemer template molecules are covalently joined to an immobilized first surface primer,

c) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;

d) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction;

e) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers; and

f) sequencing the plurality of retained forward extension strands thereby generating a plurality of extended reverse sequencing primer strands, wherein individual forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

13. The method of claim 12, wherein each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest and wherein the individual library molecules further comprise any one or any combination of two or more of

(i) a universal binding sequence for a soluble forward sequencing primer,

(ii) a universal binding sequence for a soluble reverse sequencing primer,

(iii) a universal binding sequence for an immobilized first surface primer,

(iv) a universal binding sequence for an immobilized second surface primer,

(v) a universal binding sequence for a first soluble amplification primer,
(vi) a universal binding sequence for a second soluble amplification primer,
(vii) a universal binding sequence for a soluble compaction oligonucleotide,
(viii) a sample barcode sequence and/or
(ix) a unique molecular index sequence.

14. The method of claim 12, wherein individual immobilized single stranded nucleic acid concatemer template molecules generated by the rolling circle amplification reaction comprise two or more copies of a sequence of interest and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of

(i) two or more copies of a universal binding sequence for a soluble forward sequencing primer,
(ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer,
(iii) two or more copies of a universal binding sequence for an immobilized first surface primer,
(iv) two or more copies of a universal binding sequence for an immobilized second surface primer,
(v) two or more copies of a universal binding sequence for a first soluble amplification primer,
(vi) two or more copies of a universal binding sequence for a second soluble amplification primer,
(vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide,
(viii) two or more copies of a sample barcode sequence and/or
(ix) two or more copies of a unique molecular index sequence.

15. The method of claim 12, wherein the sequencing of step (c) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

16. The method of claim 12, wherein the sequencing of step (f) comprises hybridizing a plurality of soluble reverse sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

17. The method of claim 14, wherein the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety.

18. The method of claim 17, wherein at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer.

19. The method of claim 17, wherein the plurality of immobilized second surface primers have 3' OH extendible ends.

20. The method of claim 17, wherein the plurality of immobilized second surface primers have 3' non-extendible ends.

21. The method of claim 20, wherein the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

22. A method for pairwise sequencing, comprising:
    a) contacting in-solution a plurality of single-stranded circular nucleic acid library molecules to a plurality of first soluble amplification primers, a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety that can be cleaved to generate an abasic site, under a condition suitable to form a plurality of library-primer duplexes and suitable for conducting a rolling circle amplification

reaction, thereby generating a plurality of single stranded nucleic acid concatemers having at least one nucleotide with a scissile moiety;

b) distributing the rolling circle amplification reaction onto a support having a plurality of the first surface primers immobilized thereon, under a condition suitable for hybridizing one or more portions of individual single stranded concatemers to one or more immobilized first surface primers, wherein each of the first surface primers lack a nucleotide having a scissile moiety;

c) continuing the rolling circle amplification reaction on the support to generate a plurality of immobilized concatemer template molecules;

d) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;

e) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction;

f) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers; and

g) sequencing the plurality of retained forward extension strands thereby generating a plurality of extended reverse sequencing primer strands wherein individual forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

23. The method of claim 22, wherein each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest and wherein the individual library molecules further comprise any one or any combination of two or more of

(i) a universal binding sequence for a soluble forward sequencing primer,
(ii) a universal binding sequence for a soluble reverse sequencing primer,
(iii) a universal binding sequence for an immobilized first surface primer,
(iv) a universal binding sequence for an immobilized second surface primer,
(v) a universal binding sequence for a first soluble amplification primer,
(vi) a universal binding sequence for a second soluble amplification primer,
(vii) a universal binding sequence for a soluble compaction oligonucleotide,
(viii) a sample barcode sequence and/or
(ix) a unique molecular index sequence.

24. The method of claim 22, wherein individual immobilized single stranded nucleic acid concatemer template molecules generated by the rolling circle amplification reaction comprise two or more copies of a sequence of interest and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of

(i) two or more copies of a universal binding sequence for a soluble forward sequencing primer,
(ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer,
(iii) two or more copies of a universal binding sequence for an immobilized first surface primer,
(iv) two or more copies of a universal binding sequence for an immobilized second surface primer,
(v) two or more copies of a universal binding sequence for a first soluble amplification primer,

(vi) two or more copies of a universal binding sequence for a second soluble amplification primer,

(vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide,

(viii) two or more copies of a sample barcode sequence and/or

(ix) two or more copies of a unique molecular index sequence.

25. The method of claim 22, wherein the sequencing of step (d) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

26. The method of claim 22, wherein the sequencing of step (g) comprises hybridizing a plurality of soluble reverse sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

27. The method of claim 24, wherein the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety.

28. The method of claim 27, wherein at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer.

29. The method of claim 27, wherein the plurality of immobilized second surface primers have 3' OH extendible ends.

30. The method of claim 27, wherein the plurality of immobilized second surface primers have 3' non-extendible ends.

31. The method of claim 30, wherein the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

32. A method for pairwise sequencing, comprising:

a) providing a support having a plurality of a first surface primer immobilized thereon wherein individual first surface primers in the plurality comprise a first portion (SP1-A) and a second portion (SP1-B), and the individual first surface primers comprising a 3' extendible end and lacking a nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the first surface primer;

b) contacting the plurality of the first surface primers with a plurality of single stranded linear nucleic acid library molecules, each library molecule having at the 5' end a universal sequence (SP1-A') that binds the first portion of the immobilized first surface primer, and the library molecules each having at the 3' end a universal sequence (SP1-B') that binds the second portion of the immobilized first surface primer, wherein the contacting is conducted under a condition suitable for hybridizing individual library molecules to an immobilized first surface primer to form a circularized library molecule having a gap or nick between the 5' and 3' ends of the circularized library molecule;

c) enzymatically closing the gap or nick thereby forming individual covalently closed circular molecules that are hybridized to an immobilized first surface primer;

d) generating a plurality of immobilized single stranded nucleic acid concatemer template molecules by conducting a rolling circle amplification reaction with a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a nucleotide having a scissile moiety that can be cleaved to generate an abasic site, thereby generating a plurality of immobilized single stranded nucleic acid concatemer template molecules having at least one nucleotide with a scissile moiety, wherein individual single stranded nucleic acid concatemer template molecules are covalently joined to an immobilized first surface primer;

e) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;

f) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction;

g) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized first surface primers; and

h) sequencing the plurality of retained forward extension strands thereby generating a plurality of extended reverse sequencing primer strands, wherein individual forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

33. The method of claim 32, wherein individual linear library molecules in the plurality comprise a sequence of interest and the library molecules further comprise any one or any combination of two or more of:

(i) a universal binding sequence for a soluble forward sequencing primer,

(ii) a universal binding sequence for a soluble reverse sequencing primer,

(iii) a universal binding sequence for a first portion of an immobilized first surface primer (SP1-A),

(iv) a universal binding sequence for a second portion of an immobilized first surface primer (SP1-B),

(v) a universal binding sequence for an immobilized second surface primer,

(vi) a universal binding sequence for a first soluble amplification primer,

(vii) a universal binding sequence for a second soluble amplification primer,

(viii) a universal binding sequence for a soluble compaction oligonucleotide,

(ix) a sample barcode sequence and/or

(x) a unique molecular index sequence.

34. The method of claim 32, wherein individual immobilized single stranded nucleic acid concatemer template molecules generated by the rolling circle amplification reaction comprise two or more copies of a sequence of interest and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of

(i) two or more copies of a universal binding sequence for a soluble forward sequencing primer,

(ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer,

(iii) two or more copies of a universal binding sequence for a first portion of an immobilized first surface primer (SP1-A),

(iv) two or more copies of a universal binding sequence for a second portion of an immobilized first surface primer (SP1-B),

(v) two or more copies of a universal binding sequence for an immobilized second surface primer,

(vi) two or more copies of a universal binding sequence for a first soluble amplification primer,

(vii) two or more copies of a universal binding sequence for a second soluble amplification primer,

(viii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide,

(ix) two or more copies of a sample barcode sequence and/or

(x) two or more copies of a unique molecular index sequence.

35. The method of claim 32, wherein the sequencing of step (e) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

36. The method of claim 32, wherein the sequencing of step (h) comprises hybridizing a plurality of soluble reverse sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

37. The method of claim 32, wherein the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety.

38. The method of claim 34, wherein at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer.

39. The method of claim 34, wherein the plurality of immobilized second surface primers have 3' OH extendible ends.

40. The method of claim 34, wherein the plurality of immobilized second surface primers have 3' non-extendible ends.

41. The method of claim 40, wherein the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

42. The method of claim 32, wherein the closing the gap in the circularized library molecule comprises conducting a polymerase-catalyzed gap fill-in reaction using the immobilized first surface primer as a template molecule, and ligating the nick to form a covalently closed circular molecule, wherein individual covalently closed circular molecules are hybridized to an immobilized first surface primer.

43. The method of claim 32, wherein the closing the nick in the circularized library molecule comprises conducting a ligation reaction to form a covalently closed circular molecule, and wherein individual covalently closed circular molecules are hybridized to an immobilized first surface primer.

44. A method for pairwise sequencing, comprising:

a) providing a plurality of immobilized single stranded nucleic acid concatemer template molecules each lacking a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein individual concatemer template molecules in the plurality are immobilized to a first surface primer that is immobilized to a support, and wherein the immobilized first surface primer lacks a nucleotide having a scissile moiety;

b) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;

c) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands by conducting a primer extension reaction with a plurality of soluble amplification primers and a plurality of strand-displacing polymerases to generate a plurality of forward extension strands and a plurality of partially displaced forward extension strands that are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized amplicons, and the primer extension reaction generates a plurality of detached forward extension strands (e.g., that are not hybridized to the immobilized concatemer template molecules); and

d) sequencing the plurality of immobilized partially displaced forward extension strands thereby generating a first plurality of extended reverse sequencing primer strands, and sequencing the plurality of immobilized detached forward extension strands thereby generating a second plurality of extended reverse sequencing primer strands, wherein individual immobilized partially displaced forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon, and wherein in individual immobilized detached forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

45. The method of claim 44, wherein individual concatemer template molecules in the plurality are covalently joined to an immobilized first surface primer.

46. The method of claim 44, wherein individual concatemer template molecules in the plurality are hybridized to an immobilized first surface primer.

47. The method of claim 44, wherein individual immobilized concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of

(i) two or more copies of a universal binding sequence for a soluble forward sequencing primer,

(ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer,

(iii) two or more copies of a universal binding sequence for an immobilized first surface primer,

(iv) two or more copies of a universal binding sequence for an immobilized second surface primer,

(v) two or more copies of a universal binding sequence for a first soluble amplification primer,

(vi) two or more copies of a universal binding sequence for a second soluble amplification primer,

(vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide,

(viii) two or more copies of a sample barcode sequence and/or

(ix) two or more copies of a unique molecular index sequence.

48. The method of claim 44, wherein the sequencing of step (b) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

49. The method of claim 44, wherein the sequencing of step (d) comprises hybridizing a plurality of soluble reverse sequencing primers to the plurality of immobilized partially displaced forward extension strands and the plurality of immobilized detached extended forward sequencing primer strands, and conducting one or more sequencing reactions.

50. The method of claim 47, wherein the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety.

51. The method of claim 50, wherein at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer.

52. The method of claim 50, wherein the plurality of immobilized second surface primers have 3' OH extendible ends.

53. The method of claim 50, wherein the plurality of immobilized second surface primers have 3' non-extendible ends.

54. The method of claim 53, wherein the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

55. A method for pairwise sequencing, comprising:
    a) providing a support having a plurality of a first surface primer immobilized thereon wherein each of the first surface primers have a 3' extendible end and lack a nucleotide having a scissile moiety;
    b) generating a plurality of immobilized single stranded nucleic acid concatemer template molecules by hybridizing a plurality of single-stranded circular nucleic acid library molecules to the plurality of immobilized first surface primers and conducting a rolling circle amplification reaction with a plurality of a strand displacing polymerase, and a plurality of nucleotides which lack a nucleotide having a scissile moiety that can be cleaved to generate an abasic site, thereby generating a plurality of immobilized single stranded nucleic acid concatemer

template molecules, wherein individual single stranded nucleic acid concatemer template molecules are covalently joined to an immobilized first surface primer;

c) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;

d) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands by conducting a primer extension reaction with a plurality of soluble amplification primers and a plurality of strand-displacing polymerases to generate a plurality of forward extension strands and a plurality of partially displaced forward extension strands that are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized amplicons, and the primer extension reaction generates a plurality of detached forward extension strands (e.g., that are not hybridized to the immobilized concatemer template molecules); and

e) sequencing the plurality of immobilized partially displaced forward extension strands thereby generating a first plurality of extended reverse sequencing primer strands, and sequencing the plurality of immobilized detached forward extension strands thereby generating a second plurality of extended reverse sequencing primer strands, wherein individual immobilized partially displaced forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon, and wherein in individual immobilized detached forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

56. The method of claim 55, wherein each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest, and wherein the individual library molecules further comprise any one or any combination of two or more of

(i) a universal binding sequence for a soluble forward sequencing primer,

(ii) a universal binding sequence for a soluble reverse sequencing primer,

(iii) a universal binding sequence for an immobilized first surface primer,
(iv) a universal binding sequence for an immobilized second surface primer,
(v) a universal binding sequence for a first soluble amplification primer,
(vi) a universal binding sequence for a second soluble amplification primer,
(vii) a universal binding sequence for a soluble compaction oligonucleotide,
(viii) a sample barcode sequence and/or
(ix) a unique molecular index sequence.

57. The method of claim 55, wherein individual immobilized single stranded nucleic acid concatemer template molecules generated by the rolling circle amplification reaction comprise two or more copies of a sequence of interest, wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of

(i) two or more copies of a universal binding sequence for a soluble forward sequencing primer,
(ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer,
(iii) two or more copies of a universal binding sequence for an immobilized first surface primer,
(iv) two or more copies of a universal binding sequence for an immobilized second surface primer,
(v) two or more copies of a universal binding sequence for a first soluble amplification primer,
(vi) two or more copies of a universal binding sequence for a second soluble amplification primer,
(vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide,
(viii) two or more copies of a sample barcode sequence and/or
(ix) two or more copies of a unique molecular index sequence.

58. The method of claim 55, wherein the sequencing of step (c) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

59. The method of claim 55, wherein the sequencing of step (e) comprises hybridizing a plurality of soluble reverse sequencing primers to the plurality of immobilized partially displaced forward extension strands and the plurality of immobilized detached extended forward sequencing primer strands, and conducting one or more sequencing reactions.

60. The method of claim 57, wherein the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety.

61. The method of claim 60, wherein at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer.

62. The method of claim 60, wherein the plurality of immobilized second surface primers have 3' OH extendible ends.

63. The method of claim 60, wherein the plurality of immobilized second surface primers have 3' non-extendible ends.

64. The method of claim 63, wherein the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

65. A method for pairwise sequencing, comprising:
    a) contacting in-solution a plurality of single-stranded circular nucleic acid library molecules to a plurality of first soluble amplification primers, a plurality of a strand displacing polymerase, and a plurality of nucleotides which lacks a nucleotide having a scissile moiety that can be cleaved to generate an abasic site, under a condition suitable to form a plurality of library-primer duplexes and

suitable for conducting a rolling circle amplification reaction, thereby generating a plurality of single stranded nucleic acid concatemers;

b) distributing the rolling circle amplification reaction onto a support having a plurality of the first surface primers immobilized thereon, under a condition suitable for hybridizing one or more portions of individual single stranded concatemers to one or more immobilized first surface primers, wherein each of the first surface primers lack a nucleotide having a scissile moiety;

c) continuing the rolling circle amplification reaction on the support to generate a plurality of immobilized concatemer template molecules;

d) sequencing the plurality of immobilized concatemer template molecules thereby generating a plurality of extended forward sequencing primer strands wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;

e) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands by conducting a primer extension reaction with a plurality of a second soluble amplification primer and a plurality of strand-displacing polymerases to generate a plurality of forward extension strands and a plurality of partially displaced forward extension strands that are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized amplicons, and the primer extension reaction generates a plurality of detached forward extension strands (e.g., that are not hybridized to the immobilized concatemer template molecules); and

f) sequencing the plurality of immobilized partially displaced forward extension strands thereby generating a first plurality of extended reverse sequencing primer strands, and sequencing the plurality of immobilized detached forward extension strands thereby generating a second plurality of extended reverse sequencing primer strands, wherein individual immobilized partially displaced forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon, and wherein in individual immobilized detached forward

extension strands have two or more extended reverse sequencing primer strands hybridized thereon.

66. The method of claim 65, wherein each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest, and wherein the individual library molecules further comprise any one or any combination of two or more of

(i) a universal binding sequence for a soluble forward sequencing primer,
(ii) a universal binding sequence for a soluble reverse sequencing primer,
(iii) a universal binding sequence for an immobilized first surface primer,
(iv) a universal binding sequence for an immobilized second surface primer,
(v) a universal binding sequence for a first soluble amplification primer,
(vi) a universal binding sequence for a second soluble amplification primer,
(vii) a universal binding sequence for a soluble compaction oligonucleotide,
(viii) a sample barcode sequence and/or
(ix) a unique molecular index sequence.

67. The method of claim 65, wherein individual immobilized single stranded nucleic acid concatemer template molecules generated by the rolling circle amplification reaction comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of

(i) two or more copies of a universal binding sequence for a soluble forward sequencing primer,
(ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer,
(iii) two or more copies of a universal binding sequence for an immobilized first surface primer,
(iv) two or more copies of a universal binding sequence for an immobilized second surface primer,

(v) two or more copies of a universal binding sequence for a first soluble amplification primer,

(vi) two or more copies of a universal binding sequence for a second soluble amplification primer,

(vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide,

(viii) two or more copies of a sample barcode sequence and/or

(ix) two or more copies of a unique molecular index sequence.

68. The method of claim 65, wherein the sequencing of step (d) comprises hybridizing a plurality of soluble forward sequencing primers to the plurality of immobilized concatemer template molecules and conducting one or more sequencing reactions.

69. The method of claim 65, wherein the sequencing of step (f) comprises hybridizing a plurality of soluble reverse sequencing primers to the plurality of immobilized partially displaced forward extension strands and the plurality of immobilized detached extended forward sequencing primer strands, and conducting one or more sequencing reactions.

70. The method of claim 65, wherein the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety.

71. The method of claim 70, wherein at least one copy of the universal binding sequence for the immobilized second surface primer in the individual concatemer template molecules is hybridized to an immobilized second surface primer.

72. The method of claim 70, wherein the plurality of immobilized second surface primers have 3' OH extendible ends.

73. The method of claim 70, wherein the plurality of immobilized second surface primers have 3' non-extendible ends.

74. The method of claim 73, wherein the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

75. A method for pairwise sequencing, comprising:

a) providing a plurality of immobilized single stranded nucleic acid concatemer template molecules each comprising at least one nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein individual concatemer template molecules in the plurality are immobilized to a first surface primer that is immobilized to a support, wherein the immobilized first surface primers include a nucleotide having a scissile moiety, wherein the support further comprises a plurality of immobilized second surface primers which lack a nucleotide having a scissile moiety and have an extendible terminal 3'OH group, and wherein the immobilized concatemer template molecule comprises two or more copies of a universal binding sequence for an immobilized second surface primer (wherein the support comprises an excess of immobilized first and second surface primers compared to the number of immobilized concatemer template molecules);

b) sequencing the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;

c) removing the extended forward sequencing primer strands and retaining the immobilized concatemer template molecules;

d) generating a first plurality of immobilized forward extension strands by hybridizing at least one portion of individual immobilized concatemer template molecules to a second surface primer and conducting a primer extension reaction from the second surface primers that are hybridized to a portion of the immobilized concatemer template molecule to generate a plurality of forward extension strands having a sequence that is complementary to at least a portion of

the immobilized concatemer template molecules and are covalently joined to an immobilized second surface primer;

e) contacting the plurality of immobilized concatemer template molecules and the plurality of immobilized forward extension strands with a relaxing solution which comprises at least one chaotropic agent;

f) dissociating the at least one portion of the immobilized concatemer template molecules from the immobilized second surface primers and retaining the immobilized forward extension strands, and re-hybridizing at least one portion of the immobilized concatemer template molecules to one of the immobilized second surface primers that are not covalently joined to a forward extension strand, wherein the dissociating and re-associating comprises a temperature ramp-up, a temperature plateau, and temperature ramp-down, and washing the relaxing solution from the support;

g) contacting the re-hybridized immobilized concatemer template molecules with an amplification solution and conducting a primer extension reaction from the second surface primers that are re-hybridized to a portion of the immobilized concatemer template molecules to generate a plurality of newly synthesized forward extension strands having a sequence that is complementary to at least a portion of the immobilized concatemer template molecules and are covalently joined to an immobilized second surface primer;

h) repeating steps (e) – (g) at least once;

i) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules and the immobilized first surface primers at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites thereby generating a plurality of gap-containing nucleic acid molecules while retaining the plurality of immobilized forward extension strands and retaining the plurality of immobilized second surface primers; and

j) sequencing the plurality of retained immobilized forward extension strands with a plurality of soluble reverse sequencing primers thereby generating a plurality of extended reverse sequencing primer strands.

76. The method of claim 75, wherein individual concatemer template molecules in the plurality are covalently joined to an immobilized first surface primer.

77. The method of claim 75, wherein individual concatemer template molecules in the plurality are hybridized to an immobilized first surface primer.

78. The method of claim 75, wherein individual immobilized concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of

(i) two or more copies of a universal binding sequence for a soluble forward sequencing primer,
(ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer,
(iii) two or more copies of a universal binding sequence for an immobilized first surface primer,
(iv) two or more copies of a universal binding sequence for an immobilized second surface primer,
(v) two or more copies of a universal binding sequence for a first soluble amplification primer,
(vi) two or more copies of a universal binding sequence for a second soluble amplification primer,
(vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide,
(viii) two or more copies of a sample barcode sequence and/or
(ix) two or more copies of a unique molecular index sequence.

79. A method for pairwise sequencing, comprising:

a) providing a support having a plurality of first and second surface primers immobilized thereon, wherein the first surface primers have a scissile moiety that

can be cleaved to generate an abasic site, and wherein the second surface primers lack a nucleotide having a scissile moiety and the second surface primers have an extendible terminal 3'OH group;

b) generating a plurality of immobilized single stranded nucleic acid concatemer template molecules by hybridizing a plurality of single-stranded circular nucleic acid library molecules to the plurality of immobilized first surface primers and conducting a rolling circle amplification reaction with a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a plurality of nucleotides having a scissile moiety that can be cleaved to generate an abasic site, thereby generating a plurality of immobilized single stranded nucleic acid concatemer template molecules having at least one nucleotide with a scissile moiety, wherein individual single stranded nucleic acid concatemer template molecules are covalently joined to an immobilized first surface primer;

c) sequencing the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;

d) removing the extended forward sequencing primer strands and retaining the immobilized concatemer template molecules;

e) generating a first plurality of immobilized forward extension strands by hybridizing at least one portion of individual immobilized concatemer template molecules to a second surface primer and conducting a primer extension reaction from the second surface primers that are hybridized to a portion of the immobilized concatemer template molecule to generate a plurality of forward extension strands having a sequence that is complementary to at least a portion of the immobilized concatemer template molecules and are covalently joined to an immobilized second surface primer;

f) contacting the plurality of immobilized concatemer template molecules and the plurality of immobilized forward extension strands with a relaxing solution which comprises at least one chaotropic agent;

g) dissociating the at least one portion of the immobilized concatemer template molecules from the immobilized second surface primers and retaining the immobilized forward extension strands, and re-hybridizing at least one portion of the immobilized concatemer template molecules to one of the immobilized second surface primers that are not covalently joined to a forward extension strand, wherein the dissociating and re-associating comprises a temperature ramp-up, a temperature plateau, and temperature ramp-down, and washing the relaxing solution from the support;

h) contacting the re-hybridized immobilized concatemer template molecules with an amplification solution and conducting a primer extension reaction from the second surface primers that are re-hybridized to a portion of the immobilized concatemer template molecules to generate a plurality of newly synthesized forward extension strands having a sequence that is complementary to at least a portion of the immobilized concatemer template molecules and are covalently joined to an immobilized second surface primer;

i) repeating steps (f) – (h) at least once;

j) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules and the immobilized first surface primers at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing nucleic acid molecules while retaining the plurality of immobilized forward extension strands and retaining the plurality of immobilized second surface primers; and

k) sequencing the plurality of retained immobilized forward extension strands with a plurality of soluble reverse sequencing primers thereby generating a plurality of extended reverse sequencing primer strands.

80. The method of claim 79, wherein each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest and wherein the individual library molecules further comprise any one or any combination of two or more of

(i) a universal binding sequence for a soluble forward sequencing primer,
(ii) a universal binding sequence for a soluble reverse sequencing primer,
(iii) a universal binding sequence for an immobilized first surface primer,
(iv) a universal binding sequence for an immobilized second surface primer,
(v) a universal binding sequence for a first soluble amplification primer,
(vi) a universal binding sequence for a second soluble amplification primer,
(vii) a universal binding sequence for a soluble compaction oligonucleotide,
(viii) a sample barcode sequence and/or
(ix) a unique molecular index sequence.

81. The method of claim 79, wherein individual immobilized concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of

(i) two or more copies of a universal binding sequence for a soluble forward sequencing primer,
(ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer,
(iii) two or more copies of a universal binding sequence for an immobilized first surface primer,
(iv) two or more copies of a universal binding sequence for an immobilized second surface primer,
(v) two or more copies of a universal binding sequence for a first soluble amplification primer,
(vi) two or more copies of a universal binding sequence for a second soluble amplification primer,

(vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide,

(viii) two or more copies of a sample barcode sequence and/or

(ix) two or more copies of a unique molecular index sequence.

82. A method for pairwise sequencing, comprising:

a) contacting in-solution a plurality of single-stranded circular nucleic acid library molecules to a plurality of first soluble amplification primers, a plurality of a strand displacing polymerase, and a plurality of nucleotides which include dATP, dCTP, dGTP, dTTP and a plurality of nucleotides having a scissile moiety that can be cleaved to generate an abasic site, under a condition suitable to form a plurality of library-primer duplexes and suitable for conducting a rolling circle amplification reaction, thereby generating a plurality of single stranded nucleic acid concatemers having at least one nucleotide with a scissile moiety;

b) distributing the rolling circle amplification reaction onto a support having a plurality of the first surface primers immobilized thereon, under a condition suitable for hybridizing one or more portions of individual single stranded concatemers to one or more immobilized first surface primers, wherein the immobilized first surface primers include a nucleotide having a scissile moiety, wherein the support further comprises a plurality of immobilized second surface primers which lack a nucleotide having a scissile moiety and have an extendible terminal 3'OH group;

c) continuing the rolling circle amplification reaction on the support in the presence of a plurality of nucleotides which include a plurality of nucleotides having a scissile moiety to generate a plurality of immobilized concatemer template molecules;

d) sequencing the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;

e) removing the extended forward sequencing primer strands and retaining the immobilized concatemer template molecules;

f) generating a first plurality of immobilized forward extension strands by hybridizing at least one portion of individual immobilized concatemer template molecules to a second surface primer and conducting a primer extension reaction from the second surface primers that are hybridized to a portion of the immobilized concatemer template molecule to generate a plurality of forward extension strands having a sequence that is complementary to at least a portion of the immobilized concatemer template molecules and are covalently joined to an immobilized second surface primer;

g) contacting the plurality of immobilized concatemer template molecules and the plurality of immobilized forward extension strands with a relaxing solution which comprises at least one chaotropic agent;

h) dissociating the at least one portion of the immobilized concatemer template molecules from the immobilized second surface primers and retaining the immobilized forward extension strands, and re-hybridizing at least one portion of the immobilized concatemer template molecules to one of the immobilized second surface primers that are not covalently joined to a forward extension strand, wherein the dissociating and re-associating comprises a temperature ramp-up, a temperature plateau, and temperature ramp-down, and washing the relaxing solution from the support;

i) contacting the re-hybridized immobilized concatemer template molecules with an amplification solution and conducting a primer extension reaction from the second surface primers that are re-hybridized to a portion of the immobilized concatemer template molecules to generate a plurality of newly synthesized forward extension strands having a sequence that is complementary to at least a portion of the immobilized concatemer template molecules and are covalently joined to an immobilized second surface primer;

j) repeating steps (g) – (i) at least once;

k) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules

and the immobilized first surface primers at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing nucleic acid molecules while retaining the plurality of immobilized forward extension strands and retaining the plurality of immobilized second surface primers; and

l) sequencing the plurality of retained immobilized forward extension strands with a plurality of soluble reverse sequencing primers thereby generating a plurality of extended reverse sequencing primer strands.

83. The method of claim 82, wherein each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest and wherein the individual library molecules further comprise any one or any combination of two or more of

(i) a universal binding sequence for a soluble forward sequencing primer,
(ii) a universal binding sequence for a soluble reverse sequencing primer,
(iii) a universal binding sequence for an immobilized first surface primer,
(iv) a universal binding sequence for an immobilized second surface primer,
(v) a universal binding sequence for a first soluble amplification primer,
(vi) a universal binding sequence for a second soluble amplification primer,
(vii) a universal binding sequence for a soluble compaction oligonucleotide,
(viii) a sample barcode sequence and/or
(ix) a unique molecular index sequence.

84. The method of claim 82, wherein individual immobilized concatemer template molecules in the plurality comprise two or more copies of a sequence of interest, and wherein the individual immobilized concatemer template molecules further comprise any one or any combination of two or more of

(i) two or more copies of a universal binding sequence for a soluble forward sequencing primer,

(ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer,

(iii) two or more copies of a universal binding sequence for an immobilized first surface primer,

(iv) two or more copies of a universal binding sequence for an immobilized second surface primer,

(v) two or more copies of a universal binding sequence for a first soluble amplification primer,

(vi) two or more copies of a universal binding sequence for a second soluble amplification primer,

(vii) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide,

(viii) two or more copies of a sample barcode sequence and/or

(ix) two or more copies of a unique molecular index sequence.

85. The method of claim 1, 12, 22, 32, 44, 55, 65, 75, 79 or 82, wherein the support comprises a planar substrate which comprises glass, fused-silica, silicon, a polymer (e.g., polystyrene (PS), macroporous polystyrene (MPPS), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET)), or any combination thereof.

86. The method of claim 1, 12, 22, 32, 44, 55, 65, 75, 79 or 82, wherein the support comprises at least one hydrophilic polymer coating having a water contact angle of no more than 45 degrees, and wherein at least one of the hydrophilic polymer coatings comprising branched hydrophilic polymer having at least 4 branches.

87. The method of claim 1, 12, 22, 32, 44, 55, 65, 75, 79 or 82, wherein the 5' end of the plurality of first surface primers are immobilized to the support or immobilized to a coating on the support.

88. The method of claim 1, 12, 22, 32, 44, 55, 65, 75, 79 or 82, wherein the plurality of first surface primers comprise modified oligonucleotide molecules having 2-10 phosphorothioate linkages at their 5' ends to confer resistance to nuclease degradation.

89. The method of claim 7, 17, 27, 37, 50, 60 or 70, wherein the 5' end of the plurality of second surface primers are immobilized to the support or immobilized to a coating on the support.

90. The method of claim 7, 17, 27, 37, 50, 60 or 70, wherein the plurality of second surface primers comprise modified oligonucleotide molecules having 2-10 phosphorothioate linkages at their 5' ends to confer resistance to nuclease degradation.

91. The method of claim 1, 12, 22, 32, 75, 79 or 82, wherein the immobilized concatemer template molecules comprise at least one nucleotide having a scissile moiety which comprises uridine, 8-oxo-7,8-dihydrogunine, or deoxyinosine.

92. The method of claim 1, 12, 22, 32, 75, 79 or 82, wherein the nucleotides with a scissile moiety are located at randomly distributed positions in individual immobilized concatemer template molecules in the plurality.

93. The method of claim 1, 12, 22, 32, 75, 79 or 82, wherein 0.01 – 30% of the thymidine nucleotides in the individual immobilized concatemer template molecules are replaced with uridine.

94. The method of claim 1, 12, 22, 32, 75, 79 or 82, wherein 0.01 – 30% of the guanosine nucleotides in the individual immobilized concatemer template molecules are replaced with 8-oxo-7,8-dihydrogunine or deoxyinosine.

95. The method of claim 5, 15, 25, 35, 48, 58, 68, 75, 79 or 82 wherein the soluble forward sequencing primer comprises a 3' OH extendible end and lacks a nucleotide having a scissile moiety.

96. The method of claim 6, 16, 26, 36, 49, 59, 69, 75, 79 or 82, wherein the soluble reverse sequencing primer comprises a 3' OH extendible end and lacks a nucleotide having a scissile moiety.

97. The method of claim 4, 13, 22, 23, 33, 47, 56, 65 or 66, wherein the first soluble amplification primer comprises a 3' OH extendible end and lacks a nucleotide having a scissile moiety.

98. The method of claim 4, 13, 23, 33, 47, 56 or 66, wherein the second soluble amplification primer comprises a 3' OH extendible end and lacks a nucleotide having a scissile moiety.

99. The method of claim 1 wherein the forward sequencing of step (b) comprises, or the method of claim 12 wherein the forward sequencing of step (c) comprises, or the method of claim 22 wherein the forward sequencing of step (d) comprises , or the method of claim 32 wherein the forward sequencing of step (e) comprises, or the method of claim 44 wherein the forward sequencing of step (b) comprises, or the method of claim 55 wherein the forward sequencing of step (c) comprises, or the method of claim 65 wherein the forward sequencing of step (d) comprises, or the method of claim 75 wherein the forward sequencing of step (b), or the method of claim 79 wherein the forward sequencing of step (c) comprises, or the method of claim 82 wherein the forward sequencing of step (d) comprises:

a) contacting a plurality of sequencing polymerases to (i) a plurality of immobilized concatemer template molecules and (ii) a plurality of the soluble forward sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of complexed polymerases each comprising a sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises a immobilized concatemer template molecule hybridized to a soluble forward sequencing primer;
b) contacting the plurality of complexed sequencing polymerases with a plurality of nucleotides under a condition suitable for binding at least one nucleotide to a complexed sequencing polymerase, wherein the plurality of nucleotides

comprises at least one nucleotide analog labeled with a fluorophore and having a removable chain terminating moiety at the sugar 3' position;

c) incorporating at least one nucleotide into the 3' end of the hybridized forward sequencing primers thereby generating a plurality of nascent extended forward sequencing primers; and

d) detecting the incorporated nucleotide and identifying the nucleo-base of the incorporated nucleotide.

100. The method of claim 1 wherein the reverse sequencing of step (e) comprises, or the method of claim 12 wherein the reverse sequencing of step (f) comprises, or the method of claim 22 wherein the reverse sequencing of step (g) comprises, or the method of claim 32 wherein the reverse sequencing of step (h) comprises, or the method of claim 75 wherein the reverse sequencing of step (j), or the method of claim 79 wherein the reverse sequencing of step (k) comprises, or the method of claim 82 wherein the reverse sequencing of step (l) comprises:

a) contacting a plurality of sequencing polymerases to (i) a plurality of the retained forward extension strands and (ii) a plurality of the soluble reverse sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of complexed polymerases each comprising a sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises a retained forward extension strand hybridized to a soluble reverse sequencing primer;

b) contacting the plurality of complexed sequencing polymerases with a plurality of nucleotides under a condition suitable for binding at least one nucleotide to a complexed sequencing polymerase, wherein the plurality of nucleotides comprises at least one nucleotide analog labeled with a fluorophore and having a removable chain terminating moiety at the sugar 3' position;

c) incorporating at least one nucleotide into the 3' end of the hybridized reverse sequencing primers thereby generating a plurality of nascent extended reverse sequencing primers; and

d) detecting the incorporated nucleotide and identifying the nucleo-base of the incorporated nucleotide.

101. The method of claim 100, wherein the reverse sequencing of step (a) comprises hybridizing the plurality of soluble reverse sequencing primers to the plurality of the retained forward extension strands in the presence of a high efficiency hybridization buffer which comprises:

(i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer;

(ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer;

(iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and

(iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

102. The method of claim 44 wherein the reverse sequencing of step (d) comprises, or the method of claim 55 wherein the reverse sequencing of step (e) comprises, or the method of claim 65 wherein the reverse sequencing of step (f) comprises:

a) contacting a plurality of sequencing polymerases to (i) a plurality of the immobilized partially displaced forward extension strands, (ii) a plurality of plurality of immobilized detached extended forward sequencing primer strands, and (iii) a plurality of the soluble reverse sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of complexed polymerases each comprising a sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises a soluble reverse sequencing primer hybridized to an immobilized partially displaced forward extension strand or an immobilized detached extended forward sequencing primer strand;

b) contacting the plurality of complexed sequencing polymerases with a plurality of nucleotides under a condition suitable for binding at least one nucleotide to a complexed sequencing polymerase, wherein the plurality of nucleotides

comprises at least one nucleotide analog labeled with a fluorophore and having a removable chain terminating moiety at the sugar 3' position;

c) incorporating at least one nucleotide into the 3' end of the hybridized reverse sequencing primers thereby generating a plurality of nascent extended reverse sequencing primers; and

d) detecting the incorporated nucleotide and identifying the nucleo-base of the incorporated nucleotide.

103. The method of claim 102, wherein the reverse sequencing of step (a) comprises hybridizing the plurality of soluble reverse sequencing primers to the plurality of the retained forward extension strands in the presence of a high efficiency hybridization buffer which comprises:

(i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer;

(ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer;

(iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and

(iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

104. The method of claim 1 wherein the forward sequencing of step (b) and the reverse sequencing of step (e) comprises, or the method of claim 12 wherein the forward sequencing of step (c) and the reverse sequencing of step (f) comprises, or the method of claim 22 wherein the forward sequencing of step (d) and the reverse sequencing of step (g) comprises, or the method of claim 32 wherein the forward sequencing of step (e) and the reverse sequencing of step (h) comprises, or the method of claim 44 wherein the forward sequencing of step (b) and the reverse sequencing of step (d) comprises, or the method of claim 55 wherein the forward sequencing of step (c) and the reverse sequencing of step (e) comprises, or the method of claim 65 wherein the forward sequencing of step (d) and the reverse sequence of step (f), comprises, or the method of claim 75 wherein the forward

sequencing of step (b) and the reverse sequence of step (j), comprises, or the method of claim 79 wherein the forward sequencing of step (c) and the reverse sequence of step (k), comprises, or the method of claim 82 wherein the forward sequencing of step (d) and the reverse sequence of step (l), comprises:

1) conducting a sequencing reaction at a position on the template molecule using multivalent molecules which bind but do not incorporate;
2) conducting a sequencing reaction at the same position on the template molecule using nucleotides with incorporation; and
3) repeating steps (a) and (b) at the next position on the template molecule.

105. The method of claim 1 wherein the forward sequencing of step (b) and the reverse sequencing of step (e) comprises, or the method of claim 12 wherein the forward sequencing of step (c) and the reverse sequencing of step (f) comprises, or the method of claim 22 wherein the forward sequencing of step (d) and the reverse sequencing of step (g) comprises, or the method of claim 32 wherein the forward sequencing of step (e) and the reverse sequencing of step (h) comprises, or the method of claim 75 wherein the forward sequencing of step (b) and the reverse sequence of step (j), comprises, or the method of claim 79 wherein the forward sequencing of step (c) and the reverse sequence of step (k), comprises, or the method of claim 82 wherein the forward sequencing of step (d) and the reverse sequence of step (l), comprises:

a) contacting a plurality of a first sequencing polymerase to (i) a plurality of nucleic acid template molecules and (ii) a plurality of soluble sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each comprising a first sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises the nucleic acid template molecule hybridized to the sequencing primer, wherein (1) the plurality of nucleic acid template molecules comprise a plurality of the immobilized concatemer template molecules and the plurality of soluble primers comprise a plurality of the soluble forward sequencing primers, or wherein (2) the plurality of nucleic acid template molecules comprise a plurality of the retained

forward extension strands and the plurality of soluble sequencing primers comprise a plurality of the soluble reverse sequencing primers;

b) contacting the plurality of first complexed polymerases with a plurality of detectably labeled multivalent molecules to form a plurality of multivalent-complexed polymerases, under a condition suitable for binding complementary nucleotide units of the multivalent molecules to at least two of the plurality of first complexed polymerases thereby forming a plurality of multivalent-complexed polymerases, and the condition inhibits incorporation of the complementary nucleotide units into the sequencing primers of the plurality of multivalent-complexed polymerases, wherein individual multivalent molecules in the plurality of multivalent molecules comprise a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit;

c) detecting the plurality of multivalent-complexed polymerases; and

d) identifying the nucleo-base of the complementary nucleotide units that are bound to the plurality of first complexed polymerases in the plurality of multivalent-complexed polymerases, thereby determining the sequence of the nucleic acid template.

106. The method of claim 105, wherein the reverse sequencing of step (e) in claim 1, or the reverse sequencing of step (f) in claim 12 comprises, or the reverse sequencing of step (g) in claim 22 comprises, or the reverse sequencing of step (h) in claim 32 comprises, or the reverse sequencing of step (j) in claim 75, or the reverse sequencing of step (k) in claim 79 comprises, or the reverse sequencing of step (l) in claim 82 comprises:
hybridizing the plurality of soluble reverse sequencing primers to the plurality of the retained forward extension strands in the presence of a high efficiency hybridization buffer which comprises:

(i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer;

(ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer;

(iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and

(iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

107. The method of claim 105, further comprising:

e) dissociating the plurality of multivalent-complexed polymerases and removing the plurality of first sequencing polymerases and their bound multivalent molecules, and retaining the plurality of nucleic acid duplexes;

f) contacting the plurality of the retained nucleic acid duplexes of step (e) with a plurality of second sequencing polymerases, wherein the contacting is conducted under a condition suitable for binding the plurality of second sequencing polymerases to the plurality of the retained nucleic acid duplexes, thereby forming a plurality of second complexed polymerases each comprising a second sequencing polymerase bound to a retained nucleic acid duplex;

g) contacting the plurality of second complexed polymerases with a plurality of nucleotides, wherein the contacting is conducted under a condition suitable for binding complementary nucleotides from the plurality of nucleotides to at least two of the second complexed polymerases of step (f) thereby forming a plurality of nucleotide-complexed polymerases and the condition is suitable for promoting incorporation of the bound complementary nucleotides into the sequencing primers of the nucleotide-complexed polymerases;

h) detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases; and

i) identifying the nucleo-bases of the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

108. The method of claim 105, further comprising: forming at least one avidity complex in step (b), the method comprising:

a) binding a first sequencing primer, a first sequencing polymerase, and a first multivalent molecule to a first portion of a nucleic acid template molecule thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first sequencing polymerase; and

b) binding a second sequencing primer, a second sequencing polymerase, and the first multivalent molecule to a second portion of the same nucleic acid template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the second multivalent molecule binds to the second sequencing polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms an avidity complex.

109. The method of claim 108, wherein (i) the first sequencing primer comprises a soluble forward sequencing primer and the nucleic acid template molecule comprises an immobilized concatemer template molecule, (ii) the second sequencing primer comprises a soluble forward sequencing primer and the nucleic acid template molecule comprises the same immobilized concatemer template molecule, and (iii) the first and second sequencing primers have the same sequence.

110. The method of claim 108, wherein (i) the first sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises a retained forward extension strand, (ii) the second sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises the same retained forward extension strand, and (iii) the first and second sequencing primers have the same sequence.

111. The method of claim 108, further comprising: forming at least one avidity complex in step (b), the method comprising:

a) contacting a plurality of first sequencing polymerases and a plurality of second sequencing primers with different portions of a nucleic acid template molecule to form at least first and second complexed polymerases on the same nucleic acid template molecule;

b) contacting a plurality of multivalent molecules to the at least first and second complexed polymerases on the same nucleic acid template molecule, under conditions suitable to bind a single multivalent molecule from the plurality to the first and second complexed polymerases, wherein at least a first nucleotide unit of the single multivalent molecule is bound to the first complexed polymerase which includes a first sequencing primer hybridized to a first portion of the nucleic acid template molecule thereby forming a first binding complex, and wherein at least a second nucleotide unit of the single multivalent molecule is bound to the second complexed polymerase which includes a second sequencing primer hybridized to a second portion of the same nucleic acid template molecule thereby forming a second binding complex, wherein the contacting is conducted under a condition suitable to inhibit polymerase-catalyzed incorporation of the bound first and second nucleotide units in the first and second binding complexes, and wherein the first and second binding complexes which are bound to the same multivalent molecule forms an avidity complex;

c) detecting the first and second binding complexes on the same nucleic acid template molecule, and

d) identifying the first nucleotide unit in the first binding complex thereby determining the sequence of the first portion of the nucleic acid template molecule, and identifying the second nucleotide unit in the second binding complex thereby determining the sequence of the second portion of the same nucleic acid template molecule.

112. The method of claim 111, wherein (i) the plurality of first sequencing primers comprise a plurality of first soluble forward sequencing primers and the nucleic acid template molecule comprises an immobilized concatemer template molecule, (ii) the plurality of second sequencing primers comprise a plurality of second soluble forward sequencing primers and the nucleic acid template molecule comprises the same immobilized concatemer template molecule, and (iii) the plurality of first and second sequencing primers have the same sequence.

113. The method of claim 111, wherein (i) the plurality of first sequencing primers comprises a plurality of first soluble reverse sequencing primer and the nucleic acid template molecule comprises a retained forward extension strand, (ii) the plurality of second sequencing primers comprise a plurality of second soluble reverse sequencing primers and the nucleic acid template molecule comprises the same retained forward extension strand, and (iii) the plurality of first and second sequencing primers have the same sequence.

114. The method of claim 44 wherein the forward sequencing of step (b) and the reverse sequencing of step (d) comprises, or the method of claim 44 wherein the forward sequencing of step (c) and the reverse sequencing of step (e) comprises, or the method of claim 65 wherein the forward sequencing of step (d) and the reverse sequencing of step (f) comprises:

a) contacting a plurality of a first sequencing polymerase to (i) a plurality of nucleic acid template molecules and (ii) a plurality of soluble sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each comprising a first sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises the nucleic acid template molecule hybridized to the soluble sequencing primer, wherein (1) the plurality of nucleic acid template molecules comprise a plurality of the immobilized concatemer template molecules and the plurality of sequencing primers comprise a plurality of the soluble forward sequencing primers, or wherein (2) the plurality of nucleic acid template molecules comprise a plurality of immobilized partially displaced forward extension strands and the plurality of sequencing primers comprise a plurality of the soluble reverse sequencing primers, or wherein (3) the plurality of nucleic acid template molecules comprise a plurality of immobilized detached extended forward sequencing primer strands and the plurality of sequencing primers comprise a plurality of the soluble reverse sequencing primers;

b) contacting the plurality of first complexed polymerases with a plurality of detectably labeled multivalent molecules to form a plurality of multivalent-complexed polymerases, under a condition suitable for binding complementary

nucleotide units of the multivalent molecules to at least two of the plurality of first complexed polymerases thereby forming a plurality of multivalent-complexed polymerases, and the condition inhibits incorporation of the complementary nucleotide units into the sequencing primers of the plurality of multivalent-complexed polymerases, wherein individual multivalent molecules in the plurality of multivalent molecules comprise a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit;

c) detecting the plurality of multivalent-complexed polymerases; and

d) identifying the nucleo-base of the complementary nucleotide units that are bound to the plurality of first complexed polymerases in the plurality of multivalent-complexed polymerases, thereby determining the sequence of the nucleic acid template.

115. The method of claim 114, wherein the reverse sequencing of step (d) of claim 44 comprises, or the reverse sequencing of step (e) of claim 55 comprises, the reverse sequencing of step (f) of claim 65 comprises: hybridizing the plurality of soluble reverse sequencing primers to the plurality of immobilized partially displaced forward extension strands or the plurality of immobilized detached extended forward sequencing primer strands in the presence of a high efficiency hybridization buffer which comprises:

(i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer;

(ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer;

(iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and

(iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

116. The method of claim 114, further comprising:

e) dissociating the plurality of multivalent-complexed polymerases and removing the plurality of first sequencing polymerases and their bound multivalent molecules, and retaining the plurality of nucleic acid duplexes;

f) contacting the plurality of the retained nucleic acid duplexes of step (e) with a plurality of second sequencing polymerases, wherein the contacting is conducted under a condition suitable for binding the plurality of second sequencing polymerases to the plurality of the retained nucleic acid duplexes, thereby forming a plurality of second complexed polymerases each comprising a second sequencing polymerase bound to a retained nucleic acid duplex;

g) contacting the plurality of second complexed polymerases with a plurality of nucleotides, wherein the contacting is conducted under a condition suitable for binding complementary nucleotides from the plurality of nucleotides to at least two of the second complexed polymerases of step (f) thereby forming a plurality of nucleotide-complexed polymerases and the condition is suitable for promoting incorporation of the bound complementary nucleotides into the sequencing primers of the nucleotide-complexed polymerases;

h) detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases; and

i) identifying the nucleo-bases of the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

117. The method of claim 114, further comprising: forming at least one avidity complex in step (b), the method comprising:

a) binding a first sequencing primer, a first sequencing polymerase, and a first multivalent molecule to a first portion of a nucleic acid template molecule thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first sequencing polymerase; and

b) binding a second sequencing primer, a second sequencing polymerase, and the first multivalent molecule to a second portion of the same nucleic acid template molecule thereby forming a second binding complex, wherein a second nucleotide

unit of the second multivalent molecule binds to the second sequencing polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms an avidity complex.

118. The method of claim 117, wherein (i) the first sequencing primer comprises a soluble forward sequencing primer and the nucleic acid template molecule comprises an immobilized concatemer template molecule, (ii) the second sequencing primer comprises a soluble forward sequencing primer and the nucleic acid template molecule comprises the same immobilized concatemer template molecule, and (iii) the first and second sequencing primers have the same sequence.

119. The method of claim 117, wherein (i) the first sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises an immobilized partially displaced forward extension strand, (ii) the second sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises the same immobilized partially displaced forward extension strand, and (iii) the first and second sequencing primers have the same sequence.

120. The method of claim 117, wherein (i) the first sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises an immobilized detached extended forward sequencing primer strand, (ii) the second sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises the same immobilized detached extended forward sequencing primer strand, and (iii) the first and second sequencing primers have the same sequence.

121. The method of claim 117, further comprising: forming at least one avidity complex in step (b), the method comprising:

a) contacting a plurality of first sequencing polymerases and a plurality of second sequencing primers with different portions of a nucleic acid template molecule to form at least first and second complexed polymerases on the same nucleic acid template molecule;

b) contacting a plurality of multivalent molecules to the at least first and second complexed polymerases on the same nucleic acid template molecule, under conditions suitable to bind a single multivalent molecule from the plurality to the first and second complexed polymerases, wherein at least a first nucleotide unit of the single multivalent molecule is bound to the first complexed polymerase which includes a first sequencing primer hybridized to a first portion of the nucleic acid template molecule thereby forming a first binding complex, and wherein at least a second nucleotide unit of the single multivalent molecule is bound to the second complexed polymerase which includes a second sequencing primer hybridized to a second portion of the same nucleic acid template molecule thereby forming a second binding complex, wherein the contacting is conducted under a condition suitable to inhibit polymerase-catalyzed incorporation of the bound first and second nucleotide units in the first and second binding complexes, and wherein the first and second binding complexes which are bound to the same multivalent molecule forms an avidity complex;

c) detecting the first and second binding complexes on the same nucleic acid template molecule, and

d) identifying the first nucleotide unit in the first binding complex thereby determining the sequence of the first portion of the nucleic acid template molecule, and identifying the second nucleotide unit in the second binding complex thereby determining the sequence of the second portion of the same nucleic acid template molecule.

122. The method of claim 121, wherein (i) the plurality of first sequencing primers comprise a plurality of first soluble forward sequencing primers and the nucleic acid template molecule comprises an immobilized concatemer template molecule, (ii) the plurality of second sequencing primers comprise a plurality of second soluble forward sequencing primers and the nucleic acid template molecule comprises the same immobilized concatemer template molecule, and (iii) the plurality of first and second sequencing primers have the same sequence.

123. The method of claim 121, wherein (i) the plurality of first sequencing primers comprises a plurality of first soluble reverse sequencing primer and the nucleic acid template molecule comprises an immobilized partially displaced forward extension strand, (ii) the plurality of second sequencing primers comprise a plurality of second soluble reverse sequencing primers and the nucleic acid template molecule comprises the same immobilized partially displaced forward extension strand, and (iii) the plurality of first and second sequencing primers have the same sequence.

124. The method of claim 121, wherein (i) the plurality of first sequencing primers comprises a plurality of first soluble reverse sequencing primer and the nucleic acid template molecule comprises an immobilized detached extended forward sequencing primer strands, (ii) the plurality of second sequencing primers comprise a plurality of second soluble reverse sequencing primers and the nucleic acid template molecule comprises the same immobilized detached extended forward sequencing primer strands, and (iii) the plurality of first and second sequencing primers have the same sequence.

125. The method of claim 99, 100, 102, 107 or 116, wherein individual nucleotides in the plurality of nucleotides comprise an aromatic base, a five carbon sugar, and 1-10 phosphate groups, wherein the aromatic base of the nucleotide comprises adenine, guanine, cytosine, thymine or uracil.

126. The method of claim 125, wherein the plurality of nucleotides comprises one type of nucleotide selected from a group consisting of dATP, dGTP, dCTP and dTTP.

127. The method of claim 125, wherein the plurality of nucleotides comprises a mixture of any combination of two or more types of nucleotides selected from a group consisting of dATP, dGTP, dCTP and/or dTTP.

128. The method of claim 125, wherein at least one of the nucleotides in the plurality of nucleotides comprises a fluorescently-labeled nucleotide.

129. The method of claim 125, wherein at least one of the plurality of nucleotides lacks a fluorophore label.

130. The method of claim 99, 100, 102, 107 or 116, wherein at least one of the nucleotides in the plurality of nucleotides comprises a chain terminating moiety attached to 3'-OH sugar position via cleavable moiety, and wherein the chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group.

131. The method of claim 130, wherein:

(i) the chain terminating moieties alkyl, alkenyl, alkynyl and allyl are cleavable/removable with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ);

(ii) the chain terminating moieties aryl and benzyl are cleavable/removable with H2 Pd/C;

(iii) the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable/removable with a thiol reagent which comprises beta-mercaptoethanol or dithiothritol (DTT);

(iv) the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable/removable with a phosphine reagent which comprises Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP), or Tri(hydroxyproyl)phosphine (THPP);

(v) the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable/removable with 4-dimethylaminopyridine (4-DMAP);

(vi) the chain terminating moiety carbonate is cleavable/removable with potassium carbonate (K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH); and

(vii) the chain terminating moieties urea and silyl are cleavable with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

132. The method of claim 130, wherein at least one of the nucleotides in the plurality of nucleotides comprises a chain terminating moiety attached to 3'-OH sugar position via cleavable moiety, and wherein the chain terminating moiety comprises a 3' O-azido or a 3' O-azidomethyl group.

133. The method of claim 130, wherein:
    (i) the chain terminating moieties 3' O-azido and 3' O-azidomethyl group are cleavable/removable with a phosphine compound which comprise a derivatized tri-alkyl phosphine moiety, derivatized tri-aryl phosphine moiety, Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP); and
    (ii) the chain terminating moieties 3' O-azido and 3' O-azidomethyl group are cleavable/removable with 4-dimethylaminopyridine (4-DMAP).

134. The method of claim 104, 105, 108, 111, 114, 117 or 121, wherein individual multivalent molecules in the plurality of multivalent molecules comprises **(a)** a core; and **(b)** a plurality of nucleotide arms which comprise (i) a core attachment moiety, (ii) a spacer comprising a PEG moiety, (iii) a linker, and (iv) a nucleotide unit, wherein the core is attached to the plurality of nucleotide arms via their core attachment moiety, wherein the spacer is attached to the linker, and wherein the linker is attached to the nucleotide unit.

135. The method of claim 134, wherein the core comprises an avidin-type moiety and the core attachment moiety comprises biotin.

136. The method of claim 134, wherein the linker comprises an aliphatic chain having 2-6 subunits or an oligo ethylene glycol chain having 2-6 subunits.

137. The method of claim 134, wherein the linker further comprises an aromatic moiety.

138. The method of claim 134, wherein the nucleotide unit comprises an aromatic base, a five carbon sugar and 1-10 phosphate groups.

139. The method of claim 134, wherein the linker is attached to the nucleotide unit through the base.

140. The method of claim 134, wherein the plurality of nucleotide arms attached to the core have the same type of a nucleotide unit, and wherein the types of nucleotide unit is selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

141. The method of claim 134, wherein the plurality of multivalent molecules comprise one type of a multivalent molecule wherein each multivalent molecule in the plurality has the same type of nucleotide unit selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

142. The method of claim 134, wherein the plurality of multivalent molecules comprise a mixture of any combination of two or more types of multivalent molecules each type having nucleotide units selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

143. The method of claim 134, wherein the plurality of multivalent molecules are fluorescently-labeled multivalent molecules.

144. The method of claim 134, wherein

(i) the core of individual fluorescently-labeled multivalent molecules is attached to a fluorophore which corresponds to the nucleotide units that are attached to the nucleotide arms;

(ii) at least one of the nucleotide arms comprises a linker that is attached to a fluorophore which corresponds to the nucleotide units that are attached to the nucleotide arms; and/or

(iii) at least one of the nucleotide arms comprises a nucleotide unit that is attached to a fluorophore which corresponds to the nucleotide units that are attached to the nucleotide arms.

145. The method of claim 134, wherein the plurality of multivalent molecules lack a fluorophore.

146. The method of claim 134, wherein at least one of the multivalent molecules in the plurality of multivalent molecules comprises nucleotide units having a chain terminating moiety attached to the 3'-OH sugar position via a cleavable moiety, and wherein the chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group.

147. The method of claim 146, wherein:

(i) the chain terminating moieties alkyl, alkenyl, alkynyl and allyl are cleavable/removable with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ);

(ii) the chain terminating moieties aryl and benzyl are cleavable/removable with H2 Pd/C;

(iii) the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable/removable with a thiol reagent which comprises beta-mercaptoethanol or dithiothritol (DTT);

(iv) the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable/removable with a phosphine reagent which comprises Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP), or Tri(hydroxyproyl)phosphine (THPP);

(v) the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable/removable with 4-dimethylaminopyridine (4-DMAP);

(vi) the chain terminating moiety carbonate is cleavable/removable with potassium carbonate (K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH); and

(vii) the chain terminating moieties urea and silyl are cleavable with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

148. The method of claim 146, wherein at least one of the multivalent molecules in the plurality of multivalent molecules comprises nucleotide units having a chain terminating moiety attached to the 3'-OH sugar position via a cleavable moiety, and wherein the chain terminating moiety comprises a 3' O-azido or 3' O-azidomethyl group.

149. The method of claim 146, wherein:

(i) the chain terminating moieties 3' O-azido and 3' O-azidomethyl group are cleavable/removable with a phosphine compound which comprise a derivatized tri-alkyl phosphine moiety, derivatized tri-aryl phosphine moiety, Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP); and

(ii) the chain terminating moieties 3' O-azido and 3' O-azidomethyl are cleavable/removable with 4-dimethylaminopyridine (4-DMAP).

150. The method of claim 99, 100 or 102, wherein the plurality of sequencing polymerases in step (a) comprises a recombinant wild type DNA polymerase, and wherein the plurality of nucleotides in step (b) comprises fluorescently-labeled nucleotides having a removable chain terminating moiety at the 3' sugar position.

151. The method of claim 99, 100 or 102, wherein the plurality of sequencing polymerases in step (a) comprises a mutant DNA polymerase, and wherein the plurality of nucleotides in step (b) comprises fluorescently-labeled nucleotides having a removable chain terminating moiety at the 3' sugar position.

152. The method of claim 105 or 114, wherein the plurality of first sequencing polymerases of step (a) comprise a recombinant wild type DNA polymerase.

153. The method of claim 105 or 114, wherein the plurality of first sequencing polymerases of step (a) comprise mutant DNA polymerase.

154. The method of claim 107 or 116, wherein the plurality of second sequencing polymerases of step (f) comprise recombinant wild type DNA polymerase, and wherein the plurality of nucleotides in step (b) comprises fluorescently-labeled nucleotides having a removable chain terminating moiety at the 3' sugar position.

155. The method of claim 107 or 116, wherein the plurality of second sequencing polymerases of step (f) comprise mutant DNA polymerase, and wherein the plurality of nucleotides in step (b) comprises fluorescently-labeled nucleotides having a removable chain terminating moiety at the 3' sugar position.

156. The method of claim 1 at step (c), or the method of claim 12 step (d), or the method of claim 22 step (e), or the method of claim 32 at step (f), wherein the replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction comprises:

(i) contacting at least one extended forward sequencing primer strand with a plurality of strand displacing polymerases and a plurality of nucleotides and in the absence of soluble amplification primers, under a condition suitable to conduct a strand displacing primer extension reaction using the at least one extended forward sequencing primers strand to initiate the primer extension reaction thereby generating a forward extension strand that is covalently joined to the extended forward sequencing primers strand, wherein the forward extension strand is hybridized to the immobilized concatemer template molecule.

157. The method of claim 1 at step (c), or the method of claim 12 step (d), or the method of claim 22 step (e), or the method of claim 32 at step (f), wherein the replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid

concatemer template molecules by conducting a primer extension reaction comprises removing the plurality of extended forward sequencing primer strands by:

(i) contacting the plurality of extended forward sequencing primer strands with a 5' to 3' double-stranded DNA exonuclease;

(ii) contacting the plurality of extended forward sequencing primer strands with a denaturation reagent comprising any combination of formamide, acetonitrile, guanidinium chloride and/or a pH buffering agent; or

(iii) contacting the plurality of extended forward sequencing primer strands with 100% formamide.

158. The method of claim 1 at step (c), or the method of claim 12 step (d), or the method of claim 22 step (e), or the method of claim 32 at step (f), wherein the replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction comprises:

(i) removing the plurality of extended forward sequencing primer strands while retaining the immobilized concatemer template molecules; and

(ii) contacting the plurality of retained immobilized concatemer molecules with a second plurality of soluble forward sequencing primers, a plurality of nucleotides and a plurality of primer extension polymerases, under a condition suitable to hybridize the plurality of soluble forward sequencing primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed primer extension reactions thereby generating a plurality of forward extension strands,

wherein the plurality of nucleotides comprise dATP, dGTP, dCTP and dTTP but lacks dUTP,

wherein in the plurality of primer extension polymerases are tolerant of uridine-containing template strands, and

wherein the soluble sequencing primers hybridize with the forward sequencing primer binding sequence in the retained immobilized concatemer molecules.

159. The method of claim 158, wherein the contacting comprises: contacting the plurality of retained immobilized concatemer molecules with the plurality of soluble forward sequencing primers in the presence of a high efficiency hybridization buffer which comprises:

(i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer;

(ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer;

(iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and

(iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

160. The method of claim 1 at step (c), or the method of claim 12 step (d), or the method of claim 22 step (e), or the method of claim 32 at step (f), wherein the replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction comprises:

(i) removing the plurality of extended forward sequencing primer strand while retaining the immobilized concatemer template molecules; and

(ii) contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble amplification primers, a plurality of nucleotides and a plurality of primer extension polymerases, under a condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed primer extension reactions thereby generating a plurality of forward extension strands, wherein the soluble amplification primers hybridize with the soluble amplification primer binding sequence in the retained immobilized concatemer molecules,

wherein the plurality of nucleotides comprise dATP, dGTP, dCTP and dTTP but lacks dUTP,

wherein in the plurality of primer extension polymerases are tolerant of uridine-containing template strands, and

wherein the soluble sequencing primers hybridize with the forward sequencing primer binding sequence in the retained immobilized concatemer molecules.

161. The method of claim 160 wherein the contacting comprises: contacting the plurality of retained immobilized concatemer molecules with the plurality of soluble amplification primers in the presence of a high efficiency hybridization buffer which comprises:

(i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer;

(ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer;

(iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and

(iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

162. The method of claim 156, 158 or 160, further comprising: contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble compaction oligonucleotides.

163. The method of claim 44 at step (c), or the method of claim 55 at step (d), or the method of claim 65 at step (e), wherein replacing the plurality of extended forward sequencing primer strands comprises:

(i) contacting at least one extended forward sequencing primer strand with a plurality of strand displacing polymerases and a plurality of nucleotides and in the absence of soluble amplification primers, under a condition suitable to conduct a strand displacing primer extension reaction using the at least one extended forward

sequencing primer strand to initiate the primer extension reaction thereby generating a plurality of forward extension strands, a plurality of partially displaced extended forward sequencing strands and a plurality of detached extended forward sequencing primer strands.

164. The method of claim 44 at step (c), or the method of claim 55 at step (d), or the method of claim 65 at step (e), wherein replacing the plurality of extended forward sequencing primer strands comprises: comprises removing the plurality of extended forward sequencing primer strands by:
    (i) contacting the plurality of extended forward sequencing primer strands with a 5' to 3' double-stranded DNA exonuclease;
    (ii) contacting the plurality of extended forward sequencing primer strands with a denaturation reagent comprising any combination of formamide, acetonitrile, guanidinium chloride and/or a pH buffering agent; or
    (iii) contacting the plurality of extended forward sequencing primer strands with 100% formamide.

165. The method of claim 44 at step (c), or the method of claim 55 at step (d), or the method of claim 65 at step (e), wherein replacing the plurality of extended forward sequencing primer strands comprises:
    (i) removing the plurality of extended forward sequencing primer strands while retaining the immobilized concatemer template molecules; and
    (ii) contacting the plurality of retained immobilized concatemer molecules with a second plurality of soluble forward sequencing primers, a plurality of nucleotides and a plurality of strand displacing polymerases, under a condition suitable to hybridize the plurality of soluble forward sequencing primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed strand displacing reactions thereby generating a plurality of forward extension strands and a plurality of partially displaced extended forward sequencing strands that are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized amplicons, and the primer extension

reaction generates a plurality of detached extended forward sequencing primer strands (e.g., that are not hybridized to the immobilized concatemer template molecules),

wherein the plurality of nucleotides comprise dATP, dGTP, dCTP and dTTP but lacks dUTP, and

wherein the soluble forward sequencing primers hybridize with the forward sequencing primer binding sequence in the retained immobilized concatemer molecules.

166. The method of claim 165, wherein the contacting comprises: contacting the plurality of retained immobilized concatemer molecules with the plurality of soluble forward sequencing primers in the presence of a high efficiency hybridization buffer which comprises:

(i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer;

(ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer;

(iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and

(iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

167. The method of claim 44 at step (c), or the method of claim 55 at step (d), or the method of claim 65 at step (e), wherein replacing the plurality of extended forward sequencing primer strands comprises:

(i) removing the plurality of extended forward sequencing primer strand while retaining the immobilized concatemer template molecules; and

(ii) contacting the plurality of retained immobilized concatemer molecules with a plurality of soluble amplification primers, a plurality of nucleotides and a plurality of strand displacing polymerases, under a condition suitable to hybridize the plurality of soluble amplification primers to the plurality of

retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed strand displacing reactions thereby generating a plurality of forward extension strands and a plurality of partially displaced extended forward sequencing strands that are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized amplicons, and the primer extension reaction generates a plurality of detached extended forward sequencing primer strands (e.g., that are not hybridized to the immobilized concatemer template molecules),

wherein the plurality of nucleotides comprise dATP, dGTP, dCTP and dTTP but lacks dUTP,

wherein the soluble amplification primers hybridize with the soluble amplification primer binding sequence in the retained immobilized concatemer molecules.

168. The method of claim 167, wherein the contacting comprises: contacting the plurality of retained immobilized concatemer molecules with the plurality of soluble amplification primers in the presence of a high efficiency hybridization buffer which comprises:

(i) a first polar aprotic solvent which comprises acetonitrile at 25-50% by volume of the hybridization buffer;

(ii) a second polar aprotic solvent which comprises formamide at 5-10% by volume of the hybridization buffer;

(iii) a pH buffering system which comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and

(iv) a crowding agent which comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer.

169. The method of claim 1, 12, 22, 32, 75, 79 or 82, wherein the at least one of the retained immobilized concatemer template molecules includes one or more nucleotides having a scissile moiety, and wherein the scissile moiety comprises uridine or 8-oxo-7,8-dihydroguanine, or deoxyinosine.

170. The method of claim 169, wherein the retained immobilized concatemer template molecule comprises one or more uridines, and wherein the generating the abasic sites at the uridines comprises contacting the retained immobilized concatemer template molecule with uracil DNA glycosylase (UDG).

171. The method of claim 169, wherein the retained immobilized concatemer template molecule comprises one or more 8oxoG, and wherein the generating the abasic sites at the 8oxoG comprises contacting the retained immobilized concatemer template molecule with an Fpg enzyme (formamidopyrimidine DNA glycosylase).

172. The method of claim 169, wherein the retained immobilized concatemer template molecule comprises one or more deoxyinosine, and wherein the generating the abasic sites at the deoxyinosine comprises contacting the retained immobilized concatemer template molecule with an AlkA glycosylase enzyme.

173. The method of claim 170, 171 or 172, further comprising generating a gap at the abasic sites to generate at least one gap-containing concatemer template molecule, which comprises: contacting the retained immobilized template molecules containing one or more abasic sites with an endonuclease IV, AP lyase (e.g., DNA-apurinic lyase or DNA-apyrimidinic lyase), FPG glycosylase/AP lyase and/or endo VIII glycosylase/AP lyase.

174. The method of claim 150, wherein the immobilized concatemer template molecules comprise 0.1 – 30% uridine, and wherein the plurality of wild type sequencing polymerases yield an error rate of incorporating dUTP of at least 0.1X compared to an error rate of incorporating dTTP.

175. The method of claim 151, wherein the immobilized concatemer template molecules comprise 0.1 – 30% uridine, and wherein the plurality of mutant sequencing polymerases yield an error rate of incorporating dUTP of at least 0.1X compared to an error rate of incorporating dTTP.

176. The method of claim 154, wherein the immobilized concatemer template molecules comprise 0.1 – 30% uridine, and wherein the plurality of wild type sequencing polymerases yield an error rate of incorporating dUTP of at least 0.1X compared to an error rate of incorporating dTTP.

177. The method of claim 155, wherein the immobilized concatemer template molecules comprise 0.1 – 30% uridine, and wherein the plurality of mutant sequencing polymerases yield an error rate of incorporating dUTP of at least 0.1X compared to an error rate of incorporating dTTP.

178. The method of claim 1, 12, 22, 32, 44, 55, 65, 75, 79 or 82, wherein the ratio of a first base fluorescent signal of R2 (e.g., reverse sequencing) to a first base fluorescent signal of R1 (e.g., forward sequencing) is at least 0.7 for sequencing using 1, 2, 3 or 4 dyes colors.

179. The method of claim 12, wherein the rolling circle amplification of step (b) comprises a plurality of compaction oligonucleotides and/or hexamine to generate immobilized concatemer template molecules having a more compact size and/or shape compared to a rolling circle amplification reaction in the absence of compaction oligonucleotides and/or hexamine.

180. The method of claim 12, wherein the primer extension reaction of step (d) comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of forward extension strands having a more compact size and/or shape compared to a primer extension reaction in the absence of compaction oligonucleotides and/or hexamine.

181. The method of claim 22, wherein the rolling circle amplification of step (a), (b) and/or (c) comprises a plurality of compaction oligonucleotides and/or hexamine to generate concatemer molecules having a more compact size and/or shape compared to a rolling circle amplification reaction in the absence of compaction oligonucleotides and/or hexamine.

182. The method of claim 22, wherein the primer extension reaction of step (e) comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of forward extension strands having a more compact size and/or shape compared to a primer extension reaction in the absence of compaction oligonucleotides and/or hexamine.

183. The method of claim 32, wherein the rolling circle amplification of step (d) comprises a plurality of compaction oligonucleotides and/or hexamine to generate immobilized concatemer template molecules having a more compact size and/or shape compared to a rolling circle amplification reaction in the absence of compaction oligonucleotides and/or hexamine.

184. The method of claim 32, wherein the primer extension reaction of step (f) comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of forward extension strands having a more compact size and/or shape compared to a primer extension reaction in the absence of compaction oligonucleotides and/or hexamine.

185. The method of claim 44, wherein the primer extension reaction of step (c) comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of primer extension products having a more compact size and/or shape compared to a primer extension reaction in the absence of compaction oligonucleotides and/or hexamine, wherein the plurality of primer extension products include a plurality of forward extension strands, a plurality of partially displaced extended forward sequencing strands and a plurality of detached extended forward sequencing primer strands.

186. The method of claim 55, wherein the rolling circle amplification of step (b) comprises a plurality of compaction oligonucleotides and/or hexamine to generate immobilized concatemer template molecules having a more compact size and/or shape compared to a rolling circle amplification reaction in the absence of compaction oligonucleotides and/or hexamine.

187. The method of claim 55, wherein the primer extension reaction of step (d) comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of primer

extension products having a more compact size and/or shape compared to a primer extension reaction in the absence of compaction oligonucleotides and/or hexamine, wherein the plurality of primer extension products include a plurality of forward extension strands, a plurality of partially displaced extended forward sequencing strands and a plurality of detached extended forward sequencing primer strands.

188. The method of claim 65, wherein the rolling circle amplification of steps (a), (b) and/or (c) comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of concatemer molecules having a more compact size and/or shape compared to a rolling circle amplification reaction in the absence of compaction oligonucleotides and/or hexamine.

189. The method of claim 65, wherein the primer extension reaction of step (e) comprises a plurality of compaction oligonucleotides and/or hexamine to generate a plurality of primer extension products having a more compact size and/or shape compared to a primer extension reaction in the absence of compaction oligonucleotides and/or hexamine, wherein the plurality of primer extension products include a plurality of forward extension strands, a plurality of partially displaced extended forward sequencing strands and a plurality of detached extended forward sequencing primer strands.

190. The method of claim 179, 181, 183, 186 or 188, wherein the plurality of immobilized concatemer template molecules or the plurality of immobilized concatemer molecules have FWHM (full width half maximum) of no more than about 5 μm.

191. The method of claim 180, 182 or 184, wherein the plurality of forward extension strand have FWHM (full width half maximum) of no more than about 5 μm.

192. The method of claim 185, 187 or 189, wherein the plurality of primer extension products have FWHM (full width half maximum) of no more than about 5 μm.

193. A method for pairwise sequencing, comprising:

a) providing a plurality of single stranded nucleic acid concatemer template molecules immobilized to a support;

b) sequencing the plurality of immobilized concatemer template molecules with a first plurality of sequencing polymerases, a plurality of soluble forward sequencing primers and a first plurality of multivalent molecules, thereby generating a plurality of extended forward sequencing primer strands;

c) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized concatemer template molecules by conducting a primer extension reaction;

d) removing the retained immobilized concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized surface primers; and

e) sequencing the plurality of retained forward extension strands with a second plurality of sequencing polymerases, a plurality of soluble reverse sequencing primers and a second plurality of multivalent molecules,

wherein individual multivalent molecules in the first plurality of multivalent molecules of step (b) and in the second plurality of multivalent molecules of step (e) comprise (i) a core; and (ii) a plurality of nucleotide arms which comprise a core attachment moiety, a spacer, a linker, and a nucleotide unit, wherein the core is attached to the plurality of nucleotide arms via their core attachment moiety, wherein the spacer is attached to the linker, wherein the linker is attached to the nucleotide unit.

194. The method of claim 193, wherein a nucleotide unit of an individual multivalent molecule of step (b) binds a first polymerase which is bound to a nucleic acid duplex comprising an immobilized concatemer template molecule hybridized to a forward sequencing primer.

195. The method of claim 193, wherein a nucleotide unit of an individual multivalent molecule of step (e) binds a second polymerase which is bound to a nucleic acid duplex comprising a retained forward extension strand hybridized to a reverse sequencing primer.

196. The method of claim 193, wherein the core comprises streptavidin and the core attachment moiety comprises biotin.

197. The method of claim 193, wherein in the spacer comprises a polyethylene glycol (PEG) moiety.

198. The method of claim 193, wherein the linker comprises an aliphatic chain having 2-6 subunits or an oligo ethylene glycol chain having 2-6 subunits.

199. The method of claim 193, wherein the plurality of nucleotide arms attached to the core have the same type of a nucleotide unit, and wherein the types of nucleotide unit is selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

200. The method of claim 193, wherein the first plurality of multivalent molecules of step (b) and the second plurality of multivalent molecules of step (e) comprise one type of a multivalent molecule wherein each multivalent molecule in the plurality has the same type of nucleotide unit selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

201. The method of claim 193, wherein the first plurality of multivalent molecules of step (b) and the second plurality of multivalent molecules of step (e) comprises a mixture of any combination of two or more types of multivalent molecules each type having nucleotide units selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

202. The method of claim 193, wherein at least one multivalent molecule in the first plurality of multivalent molecules of step (b) is labeled with a fluorophore, and wherein at least one

multivalent molecule in the second plurality of multivalent molecules of step (e) is labeled with a fluorophore.

203. The method of claim 193, wherein individual multivalent molecules are attached to a fluorophore that corresponds to the nucleotide units that are attached to the nucleotide arms of a given multivalent molecule.

204. The method of claim 193, wherein the sequencing of step (b), comprises:

a) contacting the first plurality of sequencing polymerases to (i) the plurality of immobilized concatemer template molecules and (ii) the plurality of soluble forward sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each comprising a first sequencing polymerase bound to a nucleic acid duplex which comprises an immobilized concatemer template molecule hybridized to a soluble forward sequencing primer;

b) contacting the plurality of first complexed polymerases with a plurality of fluorophore-labeled multivalent molecules to form a plurality of binding complexes, wherein the contacting is conducted under a condition suitable for binding complementary nucleotide units of the multivalent molecules to at least two of the plurality of first complexed polymerases thereby forming a plurality of binding complexes, and the condition inhibits incorporation of the complementary nucleotide units into the forward sequencing primers;

c) detecting the plurality of binding complexes; and

d) identifying the nucleo-base of the complementary nucleotide units that are bound to the plurality of first complexed polymerases, thereby determining the sequence of the immobilized concatemer template molecules.

205. The method of claim 204, wherein individual binding complexes in the plurality comprise a first sequencing polymerase bound to a multivalent molecule, wherein the binding complexes exhibit a persistence time of greater than 0.5 seconds.

206. The method of claim 193, further comprising: forming at least one avidity complex in step (b), the method comprising:
    a) binding a first soluble forward sequencing primer, a first forward sequencing polymerase, and a first multivalent molecule to a first portion of a first concatemer template molecule thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first sequencing polymerase; and
    b) binding a second forward sequencing primer, a second forward sequencing polymerase, and the first multivalent molecule to a second portion of the same first concatemer template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the second multivalent molecule binds to the second sequencing polymerase, and wherein the first and second binding complexes which include the same multivalent molecule forms an avidity complex.

207. The method of claim 193, wherein the plurality of immobilized concatemer template molecules comprise at least one nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein the at least one nucleotide having a scissile moiety in the immobilized concatemer template molecules which comprises uridine, 8-oxo-7,8-dihydrogunine, or deoxyinosine.

208. The method of claim 193, wherein the plurality of immobilized concatemer template molecules lack a nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein the immobilized concatemer template molecules lack a nucleotide having a scissile moiety which comprises uridine, 8-oxo-7,8-dihydrogunine, or deoxyinosine.

209. The method of claim 193, wherein individual concatemer template molecules in the plurality are covalently joined to the immobilized surface primer.

210. The method of claim 193, wherein individual concatemer template molecules in the plurality are hybridized to the immobilized surface primer.

211. The method of claim 193, wherein individual concatemer molecules in the plurality are immobilized to a surface primer which is immobilized to the support, wherein the immobilized surface primer lacks a nucleotide having a scissile moiety, and wherein the nucleotide having a scissile moiety comprises uridine, 8-oxo-7,8-dihydrogunine, or deoxyinosine.

212. A method for pairwise sequencing, comprising:

a) providing a plurality of immobilized single stranded nucleic acid concatemer template molecules each comprising at least one nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein individual concatemer template molecules in the plurality are immobilized to a surface primer that is immobilized to a support, wherein the immobilized surface primer lacks a nucleotide having a scissile moiety, and wherein the nucleotide having a scissile moiety comprises uridine;

b) sequencing the plurality of immobilized concatemer template molecules with a first plurality of sequencing polymerases, a plurality of soluble forward sequencing primers and a first plurality of multivalent molecules, thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;

c) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized concatemer template molecules by conducting a primer extension reaction;

d) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized concatemer template molecules at the nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to

generate a plurality of gap-containing concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized surface primers; and

e) sequencing the plurality of retained forward extension strands with a second plurality of sequencing polymerases, a plurality of soluble reverse sequencing primers and a second plurality of multivalent molecules, thereby generating a plurality of extended reverse sequencing primer strands, wherein individual retained forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon,

wherein individual multivalent molecules in the first plurality of multivalent molecules of step (b) and in the second plurality of multivalent molecules of step (e) comprise (i) a core; and (ii) a plurality of nucleotide arms which comprise a core attachment moiety, a spacer, a linker, and a nucleotide unit, wherein the core is attached to the plurality of nucleotide arms via their core attachment moiety, wherein the spacer is attached to the linker, wherein the linker is attached to the nucleotide unit.

213. The method of claim 212, wherein a nucleotide unit of an individual multivalent molecule of step (b) binds a first polymerase which is bound to a nucleic acid duplex comprising an immobilized concatemer template molecule hybridized to a forward sequencing primer.

214. The method of claim 212, wherein a nucleotide unit of an individual multivalent molecule of step (e) binds a second polymerase which is bound to a nucleic acid duplex comprising a retained forward extension strand hybridized to a reverse sequencing primer.

215. The method of claim 212, wherein the core comprises streptavidin and the core attachment moiety comprises biotin.

216. The method of claim 212, wherein in the spacer comprises a polyethylene glycol (PEG) moiety, and wherein the linker comprises an aliphatic chain having 2-6 subunits or an oligo ethylene glycol chain having 2-6 subunits.

217. The method of claim 212, wherein the plurality of nucleotide arms attached to the core have the same type of a nucleotide unit, and wherein the types of nucleotide unit is selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

218. The method of claim 212, wherein the first plurality of multivalent molecules of step (b) and the second plurality of multivalent molecules of step (e) comprises one type of a multivalent molecule wherein each multivalent molecule in the plurality has the same type of nucleotide unit selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

219. The method of claim 212, wherein the first plurality of multivalent molecules of step (b) and the second plurality of multivalent molecules of step (e) comprises a mixture of any combination of two or more types of multivalent molecules each type having nucleotide units selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

220. The method of claim 212, wherein at least one multivalent molecule in the first plurality of multivalent molecules of step (b) is labeled with a fluorophore, and wherein at least one multivalent molecule in the second plurality of multivalent molecules of step (e) is labeled with a fluorophore.

221. The method of claim 212, wherein the generating the abasic sites at the uridines at step (d) comprises contacting the immobilized concatemer template molecules with uracil DNA glycosylase (UDG).

222. The method of claim 212, wherein generating the plurality of gap-containing single stranded nucleic acid concatemer template molecules of step (d) comprises contacting the retained immobilized template molecules containing one or more abasic sites with an endonuclease IV, AP lyase (e.g., DNA-apurinic lyase or DNA-apyrimidinic lyase), FPG glycosylase/AP lyase and/or endo VIII glycosylase/AP lyase.

223. A method for pairwise sequencing, comprising:

a) providing a plurality of single stranded nucleic acid concatemer template molecules immobilized to a support, wherein individual concatemer template molecules in the plurality are immobilized to a surface primer where the surface primer is immobilized to the support;

b) sequencing the plurality of immobilized concatemer template molecules with a first plurality of sequencing polymerases, a plurality of soluble forward sequencing primers and a first plurality of multivalent molecules, thereby generating a plurality of extended forward sequencing primer strands;

c) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction;

d) removing the retained immobilized concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized surface primers; and

e) sequencing the plurality of retained forward extension strands with a second plurality of sequencing polymerases, a plurality of soluble reverse sequencing primers and a second plurality of multivalent molecules,

wherein the support comprises at least one hydrophilic polymer coating layer and a plurality of surface primers immobilized to the at least one hydrophilic polymer coating layer, and wherein the at least one hydrophilic polymer coating layer has a water contact angle of no more than 45 degrees.

224. The method of claim 223, wherein the at least one hydrophilic polymer coating layer comprises a molecule selected from a group consisting of polyethylene glycol (PEG), poly(vinyl alcohol) (PVA), poly(vinyl pyridine), poly(vinyl pyrrolidone) (PVP), poly(acrylic acid) (PAA), polyacrylamide, poly(N-isopropylacrylamide) (PNIPAM), poly(methyl methacrylate) (PMA), poly(2-hydroxylethyl methacrylate) (PHEMA),

poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), polyglutamic acid (PGA), poly-lysine, poly-glucoside, streptavidin, and dextran.

225. The method of claim 223, wherein the at least one hydrophilic polymer coating layer comprises polyethylene glycol (PEG).

226. The method of claim 223, wherein the at least one hydrophilic polymer coating layer comprises polymer molecules having a molecular weight of at least 1000 Daltons.

227. The method of claim 223, wherein the at least one hydrophilic polymer coating layer comprises branched polymer molecules having 4-8 branches.

228. The method of claim 223, wherein the support comprises one hydrophilic polymer coating layer and a plurality of surface primers at a surface density of least 1000/μm^2.

229. The method of claim 223, wherein the support comprises:
    a) a first coating layer comprising a first monolayer of hydrophilic polymer molecules tethered to the support;
    b) a second coating layer comprising a second monolayer of hydrophilic polymer molecules tethered to the first monolayer; and
    c) a third coating layer comprising a third monolayer of hydrophilic polymer molecules tethered to the second monolayer, and wherein the hydrophilic polymer molecules of the first layer, second layer or third layer comprise branched polymer layers.

230. The method of claim 223, wherein the surface primers are immobilized to the hydrophilic polymer molecules of the second monolayer or third monolayer, and the surface primers are distributed at a plurality of depths throughout the second layer or the third layer.

231. The method of claim 223, wherein one or more of the at least one hydrophilic polymer coating layers comprise a plurality of surface primers at a surface density of least $1000/\mu m^2$.

232. The method of claim 223, wherein one or more of the at least one hydrophilic polymer coating layers comprise a plurality of surface primers at a surface density of 1000 – 15,000 per μm^2.

233. The method of claim 223, wherein the hydrophilic polymer coating layer on the support exhibits a contrast-to-noise (CNR) ratio of at least 20 when a fluorescent image of the support is obtained by contacting the support with a fluorescently-labeled nucleotide (Cy3-labeled nucleotide) and acquiring a fluorescence image using an inverted fluorescence microscope and a camera under non-signal saturating conditions while the support is immersed in a buffer.

234. The method of claim 223, wherein the support comprises glass or plastic.

235. The method of claim 223, wherein the support is configured on a flowcell, or an interior of a capillary lumen.

236. The method of claim 223, wherein the plurality of immobilized single stranded concatemer template molecules comprise at least one nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein the at least one nucleotide having a scissile moiety in the immobilized concatemer template molecules which comprises uridine, 8-oxo-7,8-dihydrogunine, or deoxyinosine.

237. The method of claim 223, wherein the plurality of immobilized single stranded concatemer template molecules lack a nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein the immobilized concatemer template molecules lack a nucleotide having a scissile moiety which comprises uridine, 8-oxo-7,8-dihydrogunine, or deoxyinosine.

238. The method of claim 223, wherein individual concatemer template molecules in the plurality are covalently joined to an immobilized surface primer.

239. The method of claim 223, wherein individual concatemer template molecules in the plurality are hybridized to an immobilized surface primer.

240. The method of claim 223, wherein the concatemer template molecules comprise clonally amplified nucleic acid molecules.

241. The method of claim 223, wherein the immobilized surface primer lacks a nucleotide having a scissile moiety, and wherein the nucleotide having a scissile moiety comprises uridine, 8-oxo-7,8-dihydrogunine, or deoxyinosine.

242. A method for pairwise sequencing, comprising:
    a) providing a plurality of immobilized single stranded nucleic acid concatemer template molecules each comprising at least one nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecule, wherein individual concatemer template molecules in the plurality are immobilized to a surface primer that is immobilized to a support, wherein the immobilized surface primer lacks a nucleotide having a scissile moiety, and wherein the nucleotide having a scissile moiety in the concatemer template molecules comprise uridine;
    b) sequencing the plurality of immobilized concatemer template molecules with a first plurality of sequencing polymerases, a plurality of soluble forward sequencing primers and a first plurality of multivalent molecules, thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;
    c) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a

plurality of forward extension strands that are hybridized to the retained immobilized single stranded nucleic acid concatemer template molecules by conducting a primer extension reaction;

d) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized single stranded concatemer template molecules by contacting the retained immobilized concatemer template molecules with uracil DNA glycosylase (UDG), and generating gaps at the abasic sites by contacting the abasic sites with an endonuclease IV, AP lyase (e.g., DNA-apurinic lyase or DNA-apyrimidinic lyase), FPG glycosylase/AP lyase and/or endo VIII glycosylase/AP lyase to generate a plurality of gap-containing single stranded nucleic acid concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized surface primers; and

e) sequencing the plurality of retained forward extension strands with a second plurality of sequencing polymerases, a plurality of soluble reverse sequencing primers and a second plurality of multivalent molecules, thereby generating a plurality of extended reverse sequencing primer strands, wherein individual retained forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon,

wherein the support comprises at least one hydrophilic polymer coating layer and a plurality of surface primers immobilized to the at least one hydrophilic polymer coating layer, and wherein the at least one hydrophilic polymer coating layer has a water contact angle of no more than 45 degrees.

243. The method of claim 242, wherein the at least one hydrophilic polymer coating layer comprises a molecule selected from a group consisting of polyethylene glycol (PEG), poly(vinyl alcohol) (PVA), poly(vinyl pyridine), poly(vinyl pyrrolidone) (PVP), poly(acrylic acid) (PAA), polyacrylamide, poly(N-isopropylacrylamide) (PNIPAM), poly(methyl methacrylate) (PMA), poly(2-hydroxylethyl methacrylate) (PHEMA),

poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), polyglutamic acid (PGA), poly-lysine, poly-glucoside, streptavidin, and dextran.

244. The method of claim 242, wherein the at least one hydrophilic polymer coating layer comprises polymer molecules having a molecular weight of at least 1000 Daltons.

245. The method of claim 242, wherein the at least one hydrophilic polymer coating layer comprises branched polymer molecules having 4-8 branches.

246. The method of claim 242, wherein the support comprises one hydrophilic polymer coating layer and a plurality of surface primers at a surface density of least $1000/\mu m^2$.

247. The method of claim 242, wherein the support comprises:
   a) a first coating layer comprising a first monolayer of hydrophilic polymer molecules tethered to the support;
   b) a second coating layer comprising a second monolayer of hydrophilic polymer molecules tethered to the first monolayer; and
   c) a third coating layer comprising a third monolayer of hydrophilic polymer molecules tethered to the second monolayer, and wherein the hydrophilic polymer molecules of the first layer, second layer or third layer comprise branched polymer layers.

248. The method of claim 242, wherein one or more of the at least one hydrophilic polymer coating layers comprise a plurality of surface primers at a surface density of least $1000/\mu m^2$.

249. The method of claim 242, wherein the support exhibits a contrast-to-noise (CNR) ratio of at least 20 when a fluorescent image of the support is obtained by contacting the support with a fluorescently-labeled nucleotide (Cy3-labeled nucleotide) and acquiring a fluorescence image using an inverted fluorescence microscope and a camera under non-signal saturating conditions while the support is immersed in a buffer.

250. The method of claim 242, wherein the support comprises glass or plastic.

251. The method of claim 242, wherein individual concatemer template molecules in the plurality are covalently joined to an immobilized surface primer or the individual concatemer template molecules in the plurality are hybridized to an immobilized surface primer.

252. A method for pairwise sequencing, comprising:

a) providing a plurality of immobilized single stranded nucleic acid concatemer template molecules, wherein individual concatemer template molecules in the plurality are immobilized to a first surface primer that is immobilized to a support, wherein the immobilized first surface primers lack uridine, and wherein at least one of the immobilized concatemer template molecules in the plurality comprises a uridine-containing concatemer template molecule having up to 30% of thymidines replaced with uridine;

b) sequencing the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers and (i) a plurality of a first sequencing polymerase and a plurality of multivalent molecules and (ii) a plurality of a second sequencing polymerase and a plurality of nucleotide analogs, thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;

c) removing the plurality of extended forward sequencing primer strands while retaining the immobilized concatemer template molecules, and conducting a primer extension reaction with a plurality of soluble extension primers, a plurality of nucleotides and a plurality of primer extension polymerases, thereby generating a plurality of forward extension strands that are hybridized to the retained immobilized concatemer template molecules;

d) removing the retained immobilized concatemer template molecules by generating abasic sites in the immobilized concatemer template molecules at the

nucleotide(s) having the scissile moiety and generating gaps at the abasic sites to generate a plurality of gap-containing concatemer template molecules while retaining the plurality of forward extension strands and retaining the plurality of immobilized surface primers; and

e) sequencing the plurality of retained forward extension strands with a plurality of soluble reverse sequencing primers and (i) a plurality of a first sequencing polymerase and a plurality of multivalent molecules and (ii) a plurality of a second sequencing polymerase and a plurality of nucleotide analogs, thereby generating a plurality of extended reverse sequencing primer strands, wherein individual retained forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon,

wherein individual multivalent molecules of steps (b) and (e) comprise (1) a core; and (2) a plurality of nucleotide arms which comprise (i) a core attachment moiety, (ii) a spacer, (iii) a linker, and (iv) a nucleotide unit, wherein the core is attached to the plurality of nucleotide arms via their core attachment moiety, wherein the spacer is attached to the linker, and wherein the linker is attached to the nucleotide unit.

253. The method of claim 252, wherein individual concatemer template molecules in the plurality are covalently joined to an immobilized first surface primer.

254. The method of claim 252, wherein individual concatemer template molecules in the plurality are hybridized to an immobilized first surface primer.

255. The method of claim 252, wherein at least one of the plurality of immobilized concatemer template molecules lack a uridine.

256. The method of claim 252, wherein the immobilized concatemer template molecules comprise two or more copies of the sequence of interest, and two or more copies of a universal binding sequence for a soluble amplification primer, and wherein the plurality of

soluble extension primers of step (c) comprise a plurality of soluble amplification primers that hybridize to the universal binding sequence for the soluble amplification primer.

257. The method of claim 252, wherein the immobilized concatemer template molecules comprise two or more copies of the sequence of interest, and two or copies of a universal binding sequence for a soluble forward sequencing primer, and wherein the plurality of soluble extension primers of step (c) comprise a plurality of soluble forward sequencing primers that hybridize to the universal binding sequence for the soluble forward sequencing primer.

258. The method of claim 252, wherein the plurality of first sequencing polymerases in steps (b) and (e) bind a concatemer template molecule, a soluble sequencing primer and a multivalent molecule to form a binding complex which exhibits a persistence time of greater than 0.5 seconds, wherein the nucleic acid template molecule comprises an immobilized concatemer template molecule or a retained forward extension strand, and wherein the soluble sequencing primer comprises a soluble forward sequencing primer or a soluble reverse sequencing primer.

259. The method of claim 252, wherein the core of the multivalent molecules comprises streptavidin and the core attachment moiety comprise biotin.

260. The method of claim 252, wherein in the spacer of the multivalent molecules comprises a polyethylene glycol (PEG) moiety.

261. The method of claim 252, wherein the linker of the multivalent molecules comprises an aliphatic chain having 2-6 subunits or an oligo ethylene glycol chain having 2-6 subunits.

262. The method of claim 252, wherein the plurality of nucleotide arms attached to the core of the multivalent molecules have the same type of a nucleotide unit, and wherein the types of nucleotide unit is selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

263. The method of claim 252, wherein the plurality of multivalent molecules of step (b) and (e) comprise one type of a multivalent molecule wherein each multivalent molecule in the plurality has the same type of nucleotide unit selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

264. The method of claim 252, wherein the plurality of multivalent molecules of step (b) and (e) comprise a mixture of any combination of two or more types of multivalent molecules each type having nucleotide units selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

265. The method of claim 252, wherein the plurality of multivalent molecules of step (b) and (e) comprise fluorophore-labeled multivalent molecules.

266. The method of claim 252, wherein the nucleotide analog of step (b) comprises a removable chain terminating moiety at the 3' sugar group, and wherein the nucleotide analog of step (e) comprises a removable chain terminating moiety at the 3' sugar group, wherein the removable chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, azido group, O-azidomethyl group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group, and wherein the removable chain terminating moiety is cleavable with a chemical compound to generate an extendible 3'OH moiety on the sugar group.

267. The method of claim 252, wherein the plurality of nucleotide analogs of steps (b) and (e) comprise one type of nucleotide selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

268. The method of claim 252, wherein the plurality of nucleotide analogs of steps (b) and (e) comprise a mixture of any combination of two or more types of nucleotides selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

269. The method of claim 252, wherein the plurality of nucleotide analogs of steps (b) and (e) comprise at least one fluorophore-labeled nucleotide analog.

270. The method of claim 252, wherein the sequencing of step (b), comprises:

a) contacting the plurality of a first sequencing polymerase to (i) a plurality of nucleic acid template molecules which comprise a plurality of immobilized concatemer template molecules or a plurality of retained forward extension strands, and (ii) the plurality of soluble sequencing primers which comprise a plurality of soluble forward sequencing primers or a plurality of soluble reverse sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each comprising a first sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises a nucleic acid template molecule hybridized to a soluble sequencing primer;

b) contacting the plurality of first complexed polymerases with a plurality of fluorophore-labeled multivalent molecules to form a plurality of multivalent-complexed polymerases, wherein the contacting is conducted under a condition suitable for binding complementary nucleotide units of the multivalent molecules to at least two of the plurality of first complexed polymerases thereby forming a plurality of multivalent-complexed polymerases, and the condition inhibits incorporation of the complementary nucleotide units into the hybridized sequencing primers of the plurality of multivalent-complexed polymerases;

c) detecting the plurality of multivalent-complexed polymerases; and

d) identifying the nucleo-base of the complementary nucleotide units that are bound to the plurality of first complexed polymerases in the plurality of multivalent-complexed polymerases, thereby determining the sequence of the nucleic acid template.

271. The method of claim 270, further comprising:

e) dissociating the plurality of multivalent-complexed polymerases by removing the plurality of first sequencing polymerases and their bound multivalent molecules, and retaining the plurality of nucleic acid duplexes;

f) contacting the plurality of the retained nucleic acid duplexes of step (e) with a plurality of a second sequencing polymerase, wherein the contacting is conducted under a condition suitable for binding the plurality of second sequencing polymerases to the plurality of the retained nucleic acid duplexes, thereby forming a plurality of second complexed polymerases each comprising a second sequencing polymerase bound to a retained nucleic acid duplex;

g) contacting the plurality of second complexed polymerases with a plurality of nucleotides, wherein the contacting is conducted under a condition suitable for binding complementary nucleotides to at least two of the second complexed polymerases of step (f) thereby forming a plurality of nucleotide-complexed polymerases and the condition is suitable for promoting incorporation of the bound complementary nucleotides into the hybridized sequencing primers of the nucleotide-complexed polymerases thereby generating a plurality of extended sequencing primer strands wherein the plurality of extended sequencing primer strands comprise a plurality of extended forward sequencing primer strands or a plurality of extended reverse sequencing primer strands.

272. The method of claim 252, wherein the generating the abasic sites at the uridines of the immobilized concatemer template molecules of step (d) comprises contacting the immobilized concatemer template molecule with uracil DNA glycosylase (UDG).

273. The method of claim 252, wherein generating the plurality of gap-containing concatemer template molecules of step (d) comprises contacting the retained immobilized template molecules containing one or more abasic sites with an endonuclease IV, AP lyase (e.g., DNA-apurinic lyase or DNA-apyrimidinic lyase), FPG glycosylase/AP lyase and/or endo VIII glycosylase/AP lyase.

274. The method of claim 252, wherein the support comprises at least one hydrophilic polymer coating layer and a plurality of surface primers immobilized to the at least one hydrophilic polymer coating layer, and wherein the at least one hydrophilic polymer coating layer has a water contact angle of no more than 45 degrees.

275. The method of claim 274, wherein the at least one hydrophilic polymer coating layer comprises a molecule selected from a group consisting of polyethylene glycol (PEG), poly(vinyl alcohol) (PVA), poly(vinyl pyridine), poly(vinyl pyrrolidone) (PVP), poly(acrylic acid) (PAA), polyacrylamide, poly(N-isopropylacrylamide) (PNIPAM), poly(methyl methacrylate) (PMA), poly(2-hydroxylethyl methacrylate) (PHEMA), poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), polyglutamic acid (PGA), poly-lysine, poly-glucoside, streptavidin, and dextran.

276. The method of claim 275, wherein the at least one hydrophilic polymer coating layer comprises polymer molecules having a molecular weight of at least 1000 Daltons.

277. The method of claim 275, wherein the at least one hydrophilic polymer coating layer comprises branched polymer molecules having 4-8 branches.

278. The method of claim 275, wherein the support comprises:

a) a first coating layer comprising a first monolayer of hydrophilic polymer molecules tethered to the support;

b) a second coating layer comprising a second monolayer of hydrophilic polymer molecules tethered to the first monolayer; and

c) a third coating layer comprising a third monolayer of hydrophilic polymer molecules tethered to the second monolayer, and wherein the hydrophilic polymer molecules of the first layer, second layer or third layer comprise branched polymer layers.

279. The method of claim 278, wherein the surface primers are immobilized to the hydrophilic polymer molecules of the second monolayer or third monolayer, and the surface primers are distributed at a plurality of depths throughout the second layer or the third layer.

280. The method of claim 275, wherein one or more of the at least one hydrophilic polymer coating layers comprise a plurality of surface primers at a surface density of least $1000/\mu m^2$.

281. A method for pairwise sequencing, comprising:
    a) providing a support having a plurality of surface primers immobilized thereon wherein the surface primers comprise a 3' extendible end and lack a nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the surface primers;
    b) generating a plurality of immobilized single stranded nucleic acid concatemer template molecules by hybridizing a plurality of single-stranded circular nucleic acid library molecules to the plurality of immobilized surface primers and conducting a rolling circle amplification reaction with a plurality of a strand displacing polymerase and a plurality of nucleotides, thereby generating a plurality of immobilized single stranded nucleic acid concatemer template molecules, wherein individual single stranded nucleic acid concatemer template molecules are covalently joined to an immobilized surface primer, and wherein the plurality of nucleotides lacks a nucleotide having a scissile moiety that can be cleaved to generate an abasic site in the concatemer template molecules;
    c) sequencing the plurality of immobilized concatemer template molecules with a plurality of soluble forward sequencing primers, thereby generating a plurality of extended forward sequencing primer strands, wherein individual immobilized concatemer template molecules have two or more extended forward sequencing primer strands hybridized thereon;
    d) retaining the plurality of immobilized concatemer template molecules and replacing the plurality of extended forward sequencing primer strands with a plurality of forward extension strands by conducting a primer extension reaction

with a plurality of soluble amplification primers and a plurality of strand-displacing polymerases to generate a plurality of forward extension strands and a plurality of partially displaced forward extension strands wherein the forward extension strands and the partially displaced forward extension strands are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized amplicons; and

e) sequencing the plurality of immobilized partially displaced forward extension strands with a plurality of soluble reverse sequencing primers, thereby generating a plurality of extended reverse sequencing primer strands, wherein individual immobilized partially displaced forward extension strands have two or more extended reverse sequencing primer strands hybridized thereon, and wherein the immobilized partially displaced forward extension strands remain hybridized to the retained immobilized concatemer template molecules during sequencing of step (e).

282. The method of claim 281, wherein each of the single stranded circular nucleic acid library molecules in the plurality comprises a sequence of interest, and wherein the individual library molecules further comprise any one or any combination of two or more of

(i) a universal binding sequence for a soluble forward sequencing primer,
(ii) a universal binding sequence for a soluble reverse sequencing primer,
(iii) a universal binding sequence for an immobilized surface primer,
(iv) a universal binding sequence for a first soluble amplification primer,
(v) a universal binding sequence for a second soluble amplification primer,
(vi) a universal binding sequence for a soluble compaction oligonucleotide,
(vii) a sample barcode sequence and/or
(viii) a unique molecular index sequence.

283. The method of claim 281, wherein individual immobilized single stranded nucleic acid concatemer template molecules generated by the rolling circle amplification reaction comprise two or more copies of a sequence of interest, wherein the individual immobilized

concatemer template molecules further comprise any one or any combination of two or more of

(i) two or more copies of a universal binding sequence for a soluble forward sequencing primer,

(ii) two or more copies of a universal binding sequence for a soluble reverse sequencing primer,

(iii) two or more copies of a universal binding sequence for an immobilized surface primer,

(iv) two or more copies of a universal binding sequence for a first soluble amplification primer,

(v) two or more copies of a universal binding sequence for a second soluble amplification primer,

(vi) two or more copies of a universal binding sequence for a soluble compaction oligonucleotide,

(vii) two or more copies of a sample barcode sequence and/or

(viii) two or more copies of a unique molecular index sequence.

284. The method of claim 281, wherein replacing the plurality of extended forward sequencing primer strands of step (d) comprises:

(i) removing the plurality of extended forward sequencing primer strands while retaining the immobilized concatemer template molecules; and

(ii) contacting the plurality of retained immobilized concatemer molecules with the plurality of soluble amplification primers, a plurality of nucleotides and a plurality of strand displacing polymerases, under a condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed strand displacing reactions thereby generating a plurality of forward extension strands and a plurality of partially displaced extended forward sequencing strands that are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized amplicons.

285. The method of claim 284, wherein the strand displacing polymerase comprises phi29 DNA polymerase, large fragment of Bst DNA polymerase, large fragment of Bsu DNA polymerase (exo-), Bca DNA polymerase (exo-), Klenow fragment of E. coli DNA polymerase, T5 polymerase, M-MuLV reverse transcriptase, HIV viral reverse transcriptase, Deep Vent DNA polymerase and KOD DNA polymerase.

286. The method of claim 281 wherein the forward sequencing of step (c) comprises:

a) contacting a plurality of sequencing polymerases and a plurality of the soluble forward sequencing primers to a plurality of immobilized concatemer template molecules, wherein the contacting is conducted under a condition suitable to form a plurality of complexed polymerases each comprising a sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises an immobilized concatemer template molecule hybridized to a soluble forward sequencing primer;

b) contacting the plurality of complexed sequencing polymerases with a plurality of nucleotides under a condition suitable for binding at least one nucleotide to a complexed sequencing polymerase, wherein the plurality of nucleotides comprises at least one nucleotide analog labeled with a fluorophore and having a removable chain terminating moiety at the sugar 3' position;

c) incorporating at least one nucleotide into the 3' end of the hybridized forward sequencing primers thereby generating a plurality of nascent extended forward sequencing primers; and

d) detecting the incorporated nucleotide and identifying the nucleo-base of the incorporated nucleotide.

287. The method of claim 286, wherein at least one of the nucleotides in the plurality of nucleotides of step (b) comprises a removable chain terminating moiety attached to the 3' carbon position of the sugar group, wherein the removable chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, azido group, O-azidomethyl group, amine group, amide group, keto

group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group, and wherein the removable chain terminating moiety is cleavable with a chemical compound to generate an extendible 3'OH moiety on the sugar group.

288. The method of claim 281, wherein the reverse sequencing of step (e) comprises:

a) contacting a plurality of sequencing polymerases and a plurality of the soluble reverse sequencing primers to the plurality of the immobilized partially displaced forward extension strands, wherein the contacting is conducted under a condition suitable to form a plurality of complexed polymerases each comprising a sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises a soluble reverse sequencing primer hybridized to an immobilized partially displaced forward extension strand;

b) contacting the plurality of complexed sequencing polymerases with a plurality of nucleotides under a condition suitable for binding at least one nucleotide to a complexed sequencing polymerase, wherein the plurality of nucleotides comprises at least one nucleotide analog labeled with a fluorophore and having a removable chain terminating moiety at the sugar 3' position;

c) incorporating at least one nucleotide into the 3' end of the hybridized reverse sequencing primers thereby generating a plurality of nascent extended reverse sequencing primers; and

d) detecting the incorporated nucleotide and identifying the nucleo-base of the incorporated nucleotide.

289. The method of claim 288, wherein at least one of the nucleotides in the plurality of nucleotides of step (b) comprises a removable chain terminating moiety attached to the 3' carbon position of the sugar group, wherein the removable chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, azido group, O-azidomethyl group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group, and wherein the removable chain terminating moiety is

cleavable with a chemical compound to generate an extendible 3'OH moiety on the sugar group.

290. The method of claim 281, wherein the forward sequencing of step (b) and the reverse sequencing of step (e) comprise:

a) contacting a plurality of a first sequencing polymerase and a plurality of soluble sequencing primers to a plurality of nucleic acid template molecules, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each comprising a first sequencing polymerase bound to a nucleic acid duplex which comprises the nucleic acid template molecule hybridized to the soluble sequencing primer, wherein (1) the plurality of nucleic acid template molecules comprise a plurality of the immobilized concatemer template molecules and the plurality of sequencing primers comprise a plurality of the soluble forward sequencing primers, or wherein (2) the plurality of nucleic acid template molecules comprise a plurality of immobilized partially displaced forward extension strands and the plurality of sequencing primers comprise a plurality of the soluble reverse sequencing primers;

b) contacting the plurality of first complexed polymerases with a plurality of detectably labeled multivalent molecules to form a plurality of multivalent-complexed polymerases, wherein individual multivalent molecules in the plurality of multivalent molecules comprise a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit, wherein the contacting is performed under a condition suitable for binding complementary nucleotide units of the multivalent molecules to at least two of the plurality of first complexed polymerases thereby forming a plurality of multivalent-complexed polymerases, and the condition inhibits incorporation of the complementary nucleotide units into the sequencing primers of the plurality of multivalent-complexed polymerases;

c) detecting the plurality of multivalent-complexed polymerases; and

d) identifying the nucleo-base of the complementary nucleotide units that are bound to the plurality of first complexed polymerases in the plurality of multivalent-

complexed polymerases, thereby determining the sequence of the nucleic acid template.

291. The method of claim 290, further comprising:

e) dissociating the plurality of multivalent-complexed polymerases and removing the plurality of first sequencing polymerases and their bound multivalent molecules, and retaining the plurality of nucleic acid duplexes;

f) contacting the plurality of the retained nucleic acid duplexes of step (e) with a plurality of second sequencing polymerases, wherein the contacting is conducted under a condition suitable for binding the plurality of second sequencing polymerases to the plurality of the retained nucleic acid duplexes, thereby forming a plurality of second complexed polymerases;

g) contacting the plurality of second complexed polymerases with a plurality of nucleotides under a condition suitable for binding complementary nucleotides from the plurality of nucleotides to at least two of the second complexed polymerases of step (f) thereby forming a plurality of nucleotide-complexed polymerases and the condition is suitable for promoting incorporation of the bound complementary nucleotides into the sequencing primers of the nucleotide-complexed polymerases.

292. The method of claim 291, further comprising:

h) detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

293. The method of claim 291, further comprising:

h) detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases; and

i) identifying the nucleo-bases of the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

294. The method of claim 290, further comprising: forming at least one avidity complex in step (b), the method comprising:
    a) binding a first sequencing primer, a first sequencing polymerase, and a first multivalent molecule to a first portion of a nucleic acid template molecule thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first sequencing polymerase; and
    b) binding a second sequencing primer, a second sequencing polymerase, and the first multivalent molecule to a second portion of the same nucleic acid template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the second multivalent molecule binds to the second sequencing polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms an avidity complex.

295. The method of claim 294, wherein (i) the first sequencing primer comprises a soluble forward sequencing primer and the nucleic acid template molecule comprises an immobilized concatemer template molecule, (ii) the second sequencing primer comprises a soluble forward sequencing primer and the nucleic acid template molecule comprises the same immobilized concatemer template molecule, and (iii) the first and second sequencing primers have the same sequence.

296. The method of claim 294, wherein (i) the first sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises an immobilized partially displaced forward extension strand, (ii) the second sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises the same immobilized partially displaced forward extension strand, and (iii) the first and second sequencing primers have the same sequence.

297. The method of claim 290, wherein individual multivalent molecules in the plurality of multivalent molecules comprises (a) a core; and (b) a plurality of nucleotide arms which comprise (i) a core attachment moiety, (ii) a spacer comprising a PEG moiety, (iii) a linker, and (iv) a nucleotide unit, wherein the core is attached to the plurality of nucleotide arms

via their core attachment moiety, wherein the spacer is attached to the linker, and wherein the linker is attached to the nucleotide unit.

298. The method of claim 297, wherein
    (i) the core comprises streptavidin and the core attachment moiety comprises biotin;
    (ii) the linker comprises an aliphatic chain having 2-6 subunits or an oligo ethylene glycol chain having 2-6 subunits; and
    (iii) the nucleotide unit comprises an aromatic base, a five carbon sugar and 1-10 phosphate groups.

299. The method of claim 297, wherein the plurality of nucleotide arms attached to the core have the same type of a nucleotide unit, and wherein the types of nucleotide unit is selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

300. The method of claim 297, wherein the plurality of multivalent molecules comprise one type of a multivalent molecule wherein each multivalent molecule in the plurality has the same type of nucleotide unit selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

301. The method of claim 297, wherein the plurality of multivalent molecules comprise a mixture of any combination of two or more types of multivalent molecules each type having nucleotide units selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

302. The method of claim 290, wherein the plurality of detectably labeled multivalent molecules comprise fluorescently-labeled multivalent molecules.

303. The method of claim 281, wherein the support comprises at least one hydrophilic polymer coating layer and a plurality of surface primers immobilized to the at least one hydrophilic

polymer coating layer, and wherein the at least one hydrophilic polymer coating layer has a water contact angle of no more than 45 degrees.

304. The method of claim 303, wherein the at least one hydrophilic polymer coating layer comprises a molecule selected from a group consisting of polyethylene glycol (PEG), poly(vinyl alcohol) (PVA), poly(vinyl pyridine), poly(vinyl pyrrolidone) (PVP), poly(acrylic acid) (PAA), polyacrylamide, poly(N-isopropylacrylamide) (PNIPAM), poly(methyl methacrylate) (PMA), poly(2-hydroxylethyl methacrylate) (PHEMA), poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), polyglutamic acid (PGA), poly-lysine, poly-glucoside, streptavidin, and dextran.

305. The method of claim 303, wherein the at least one hydrophilic polymer coating layer comprises polymer molecules having a molecular weight of at least 1000 Daltons.

306. The method of claim 303, wherein the at least one of the hydrophilic polymer coating layer comprises branched hydrophilic polymer molecules having 4-8 branches.

307. The method of claim 303, wherein the at least one hydrophilic polymer coating layer comprises a plurality of surface primers at a surface density of least 1000/um^2.

308. The method of claim 303, wherein the support comprises:

a) a first coating layer comprising a first monolayer of hydrophilic polymer molecules tethered to the support;

b) a second coating layer comprising a second monolayer of hydrophilic polymer molecules tethered to the first monolayer; and

c) a third coating layer comprising a third monolayer of hydrophilic polymer molecules tethered to the second monolayer, and wherein the hydrophilic polymer molecules of the first layer, second layer or third layer comprise branched polymer layers,

wherein the surface primers are immobilized to the hydrophilic polymer molecules of the second monolayer or third monolayer, and the surface primers

are distributed at a plurality of depths throughout the second layer or the third layer.

309. The method of claim 303, wherein the hydrophilic coating layer on the support exhibits a contrast-to-noise (CNR) ratio of at least 20 when a fluorescent image of the support is obtained by contacting the support with a fluorescently-labeled nucleotide (Cy3-labeled nucleotide) and acquiring a fluorescence image using an inverted fluorescence microscope and a camera under non-signal saturating conditions while the support is immersed in a buffer.

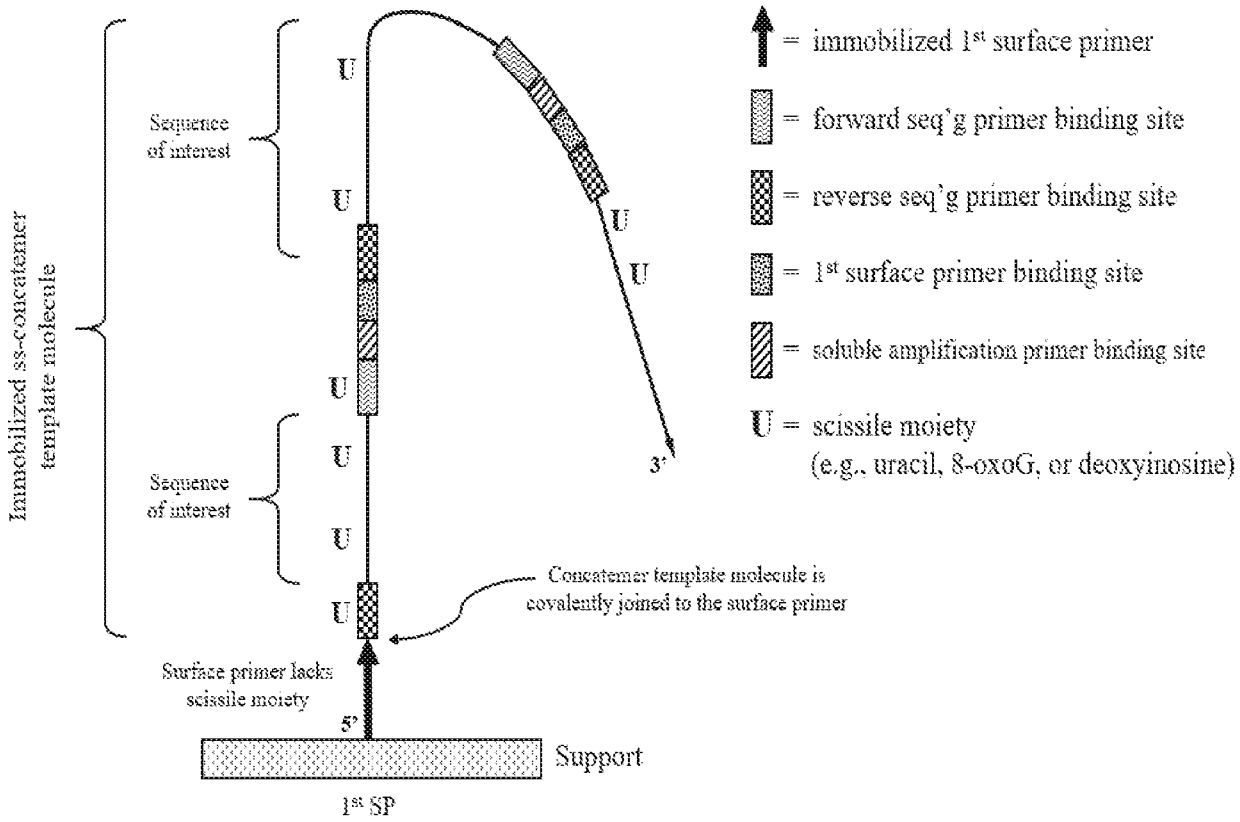

Immobilized ss-concatemer template molecule
Sequence of interest
Sequence of interest
U
U
U
U
U
U
U
3'
Concatemer template molecule is covalently joined to the surface primer
Surface primer lacks scissile moiety
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety
(e.g., uracil, 8-oxoG, or deoxyinosine)

FIG. 1

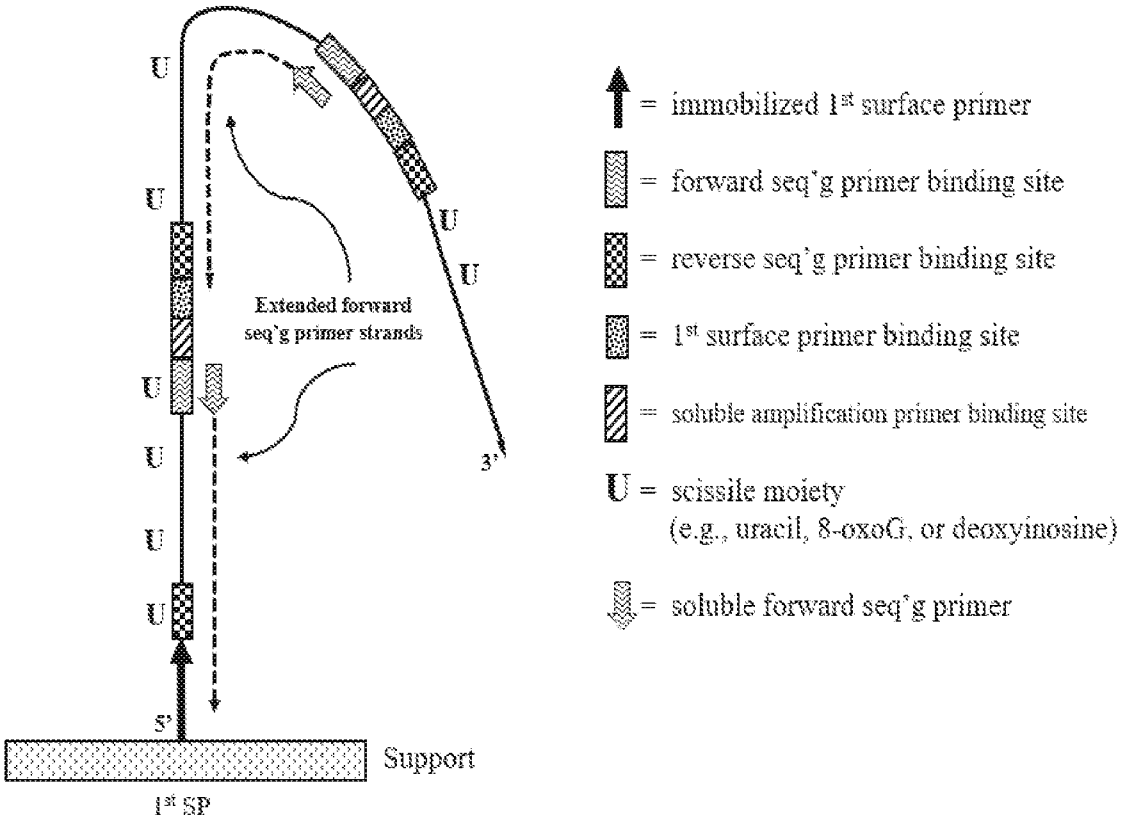

U
U
U
U
U
U
U
U
Extended forward seq'g primer strands
3'
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble forward seq'g primer

FIG. 2

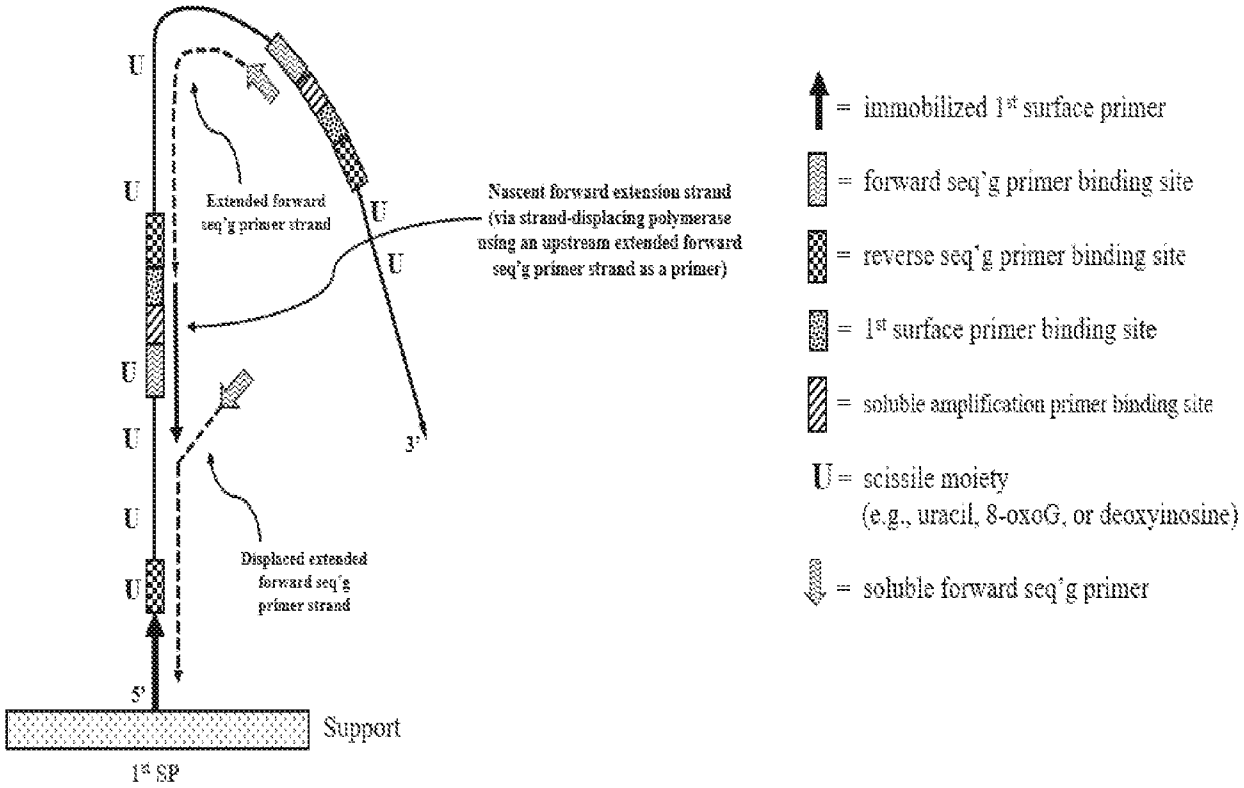

U
U
U
U
U
U
Extended forward seq'g primer strand
Nascent forward extension strand (via strand-displacing polymerase using an upstream extended forward seq'g primer strand as a primer)
3'
Displaced extended forward seq'g primer strand
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble forward seq'g primer

FIG. 3

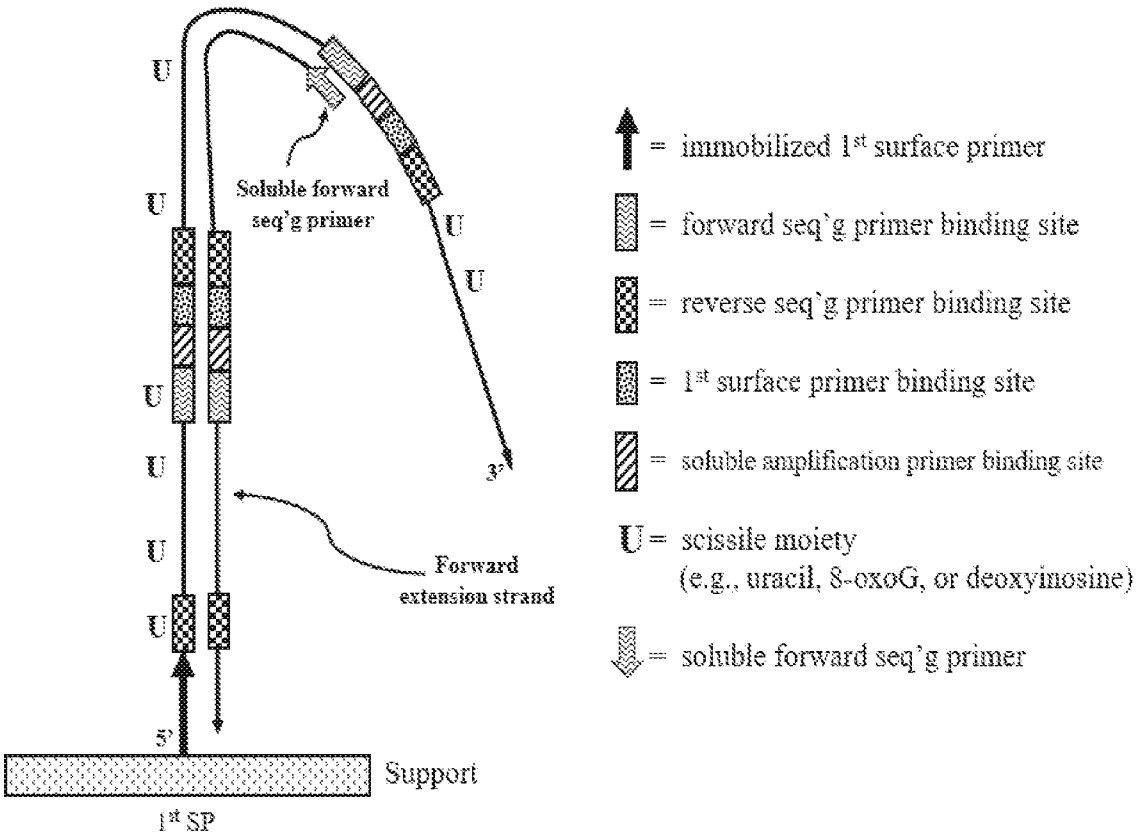

U
U
U
U
U
U
U
Soluble forward seq'g primer
U
U
3'
Forward extension strand
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq`g primer binding site
= reverse seq`g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble forward seq'g primer

FIG. 4

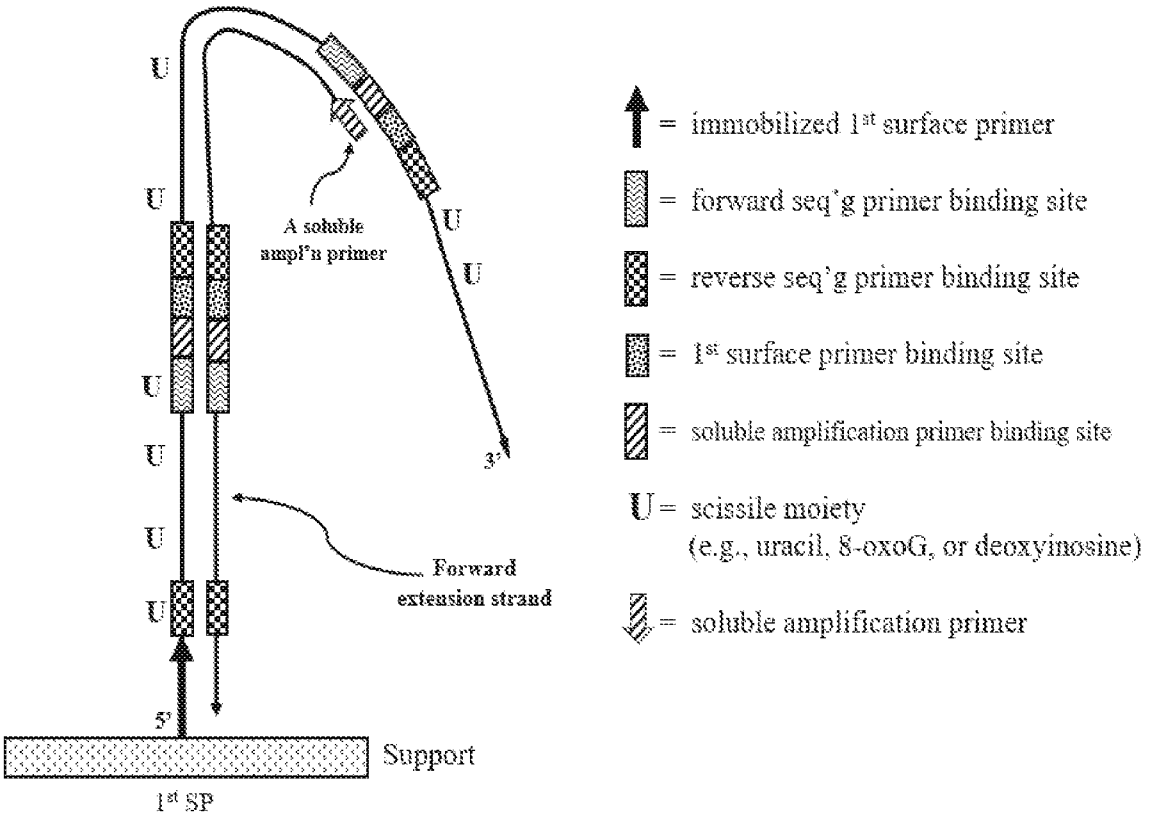

U
U
U
U
U
U
U
A soluble ampl'n primer
3'
Forward extension strand
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble amplification primer

FIG. 5

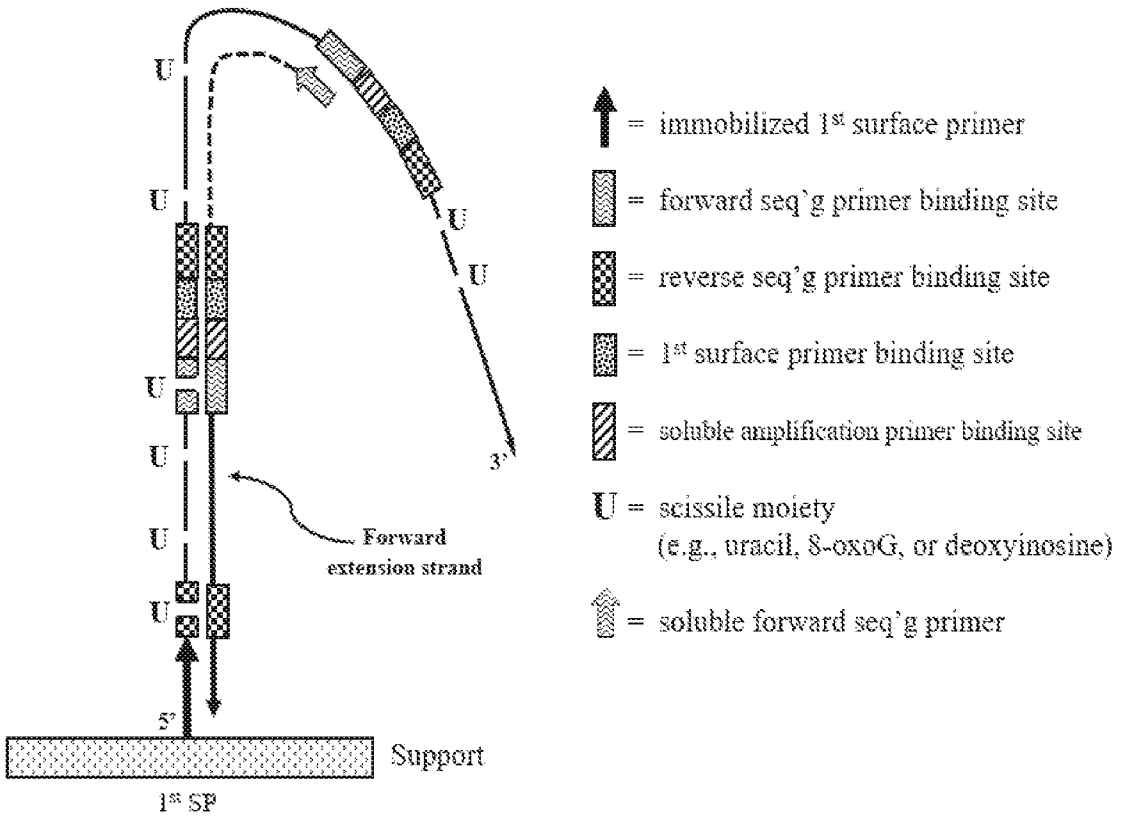

U
U
U
U
U
U
U
U
3'
Forward extension strand
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq`g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble forward seq'g primer

FIG. 6

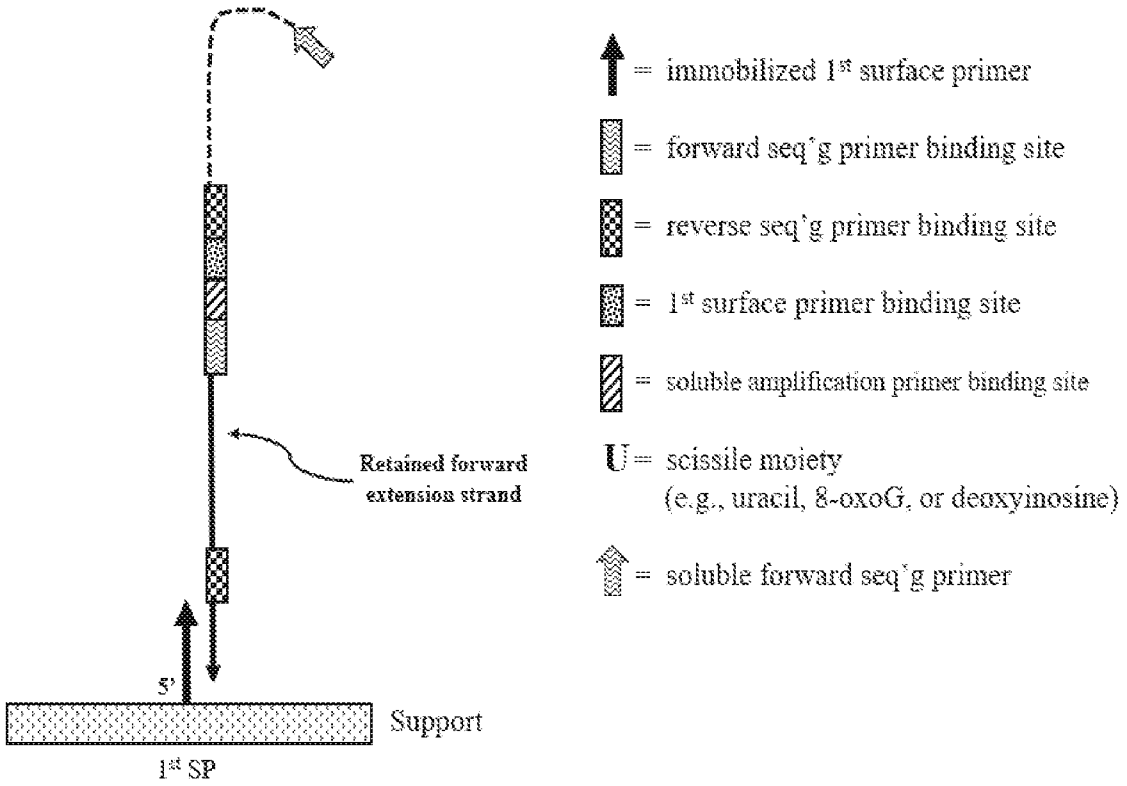

= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety
(e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble forward seq'g primer
Retained forward extension strand
5'
Support
1st SP

FIG. 7

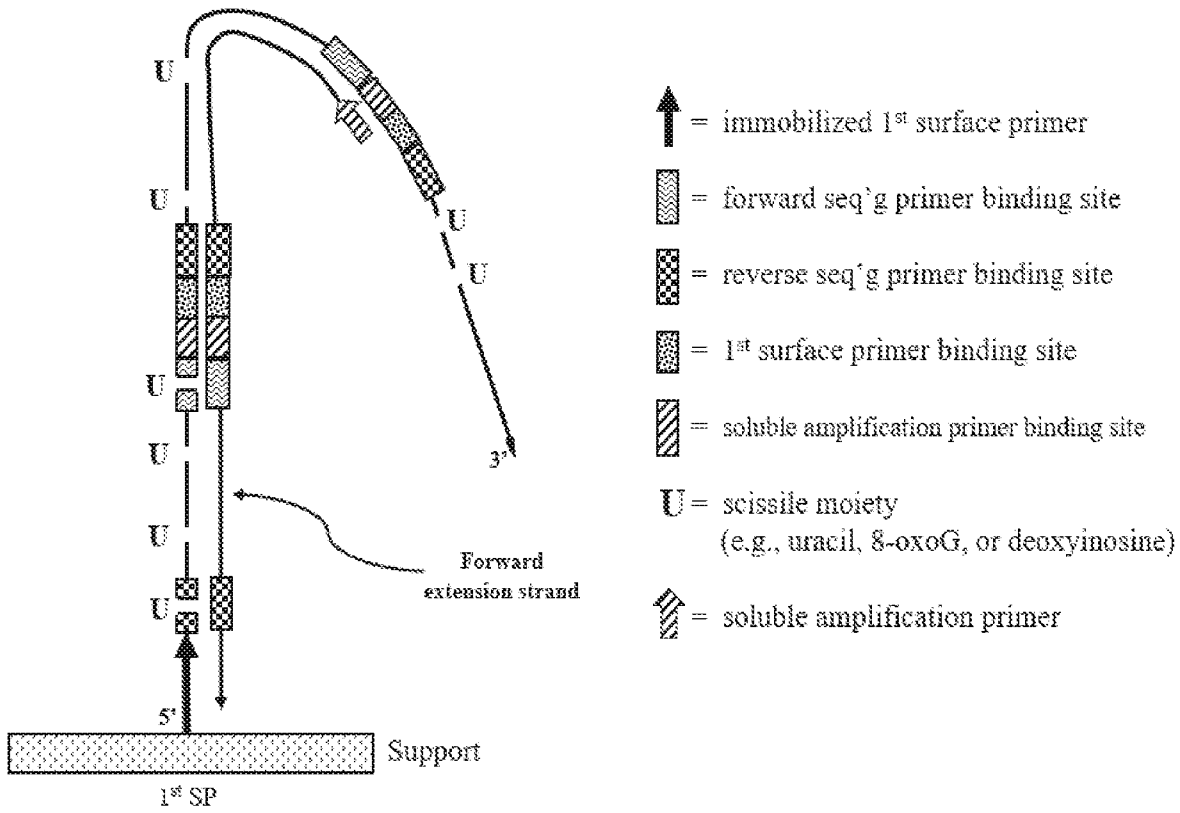

U
U
U
U
U
U
U
U
3'
Forward
extension strand
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety
(e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble amplification primer

FIG. 8

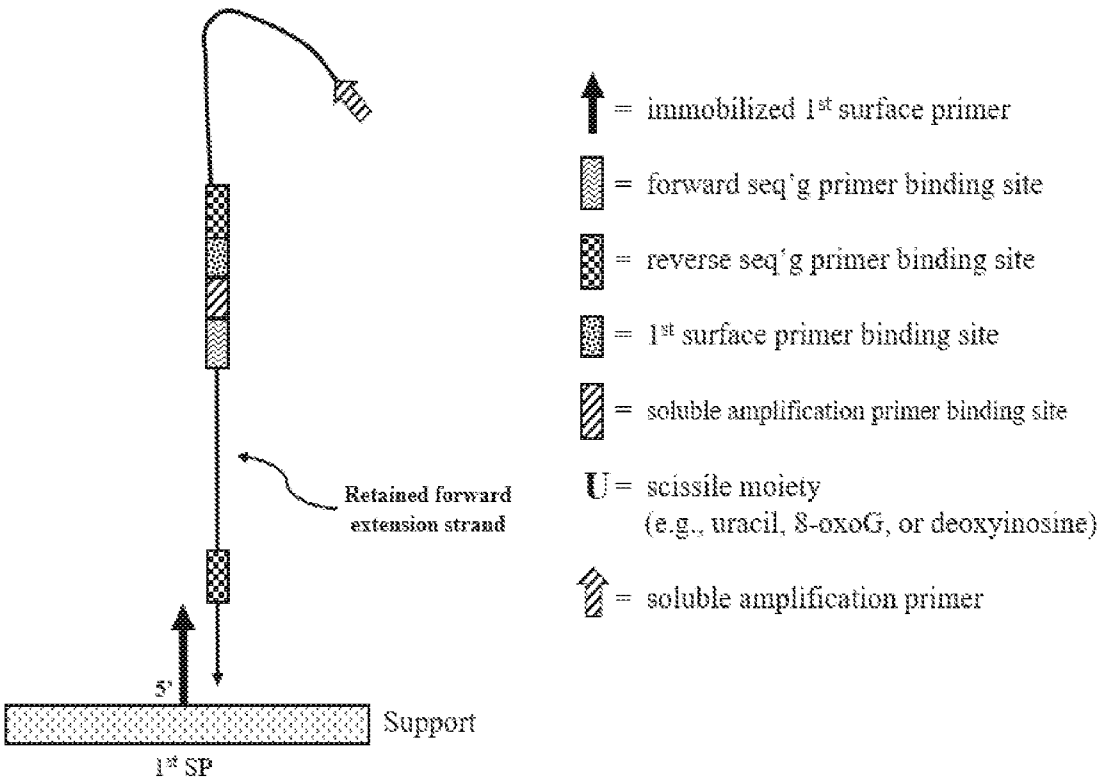

= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety
(e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble amplification primer
Retained forward extension strand
5'
Support
1st SP

FIG. 9

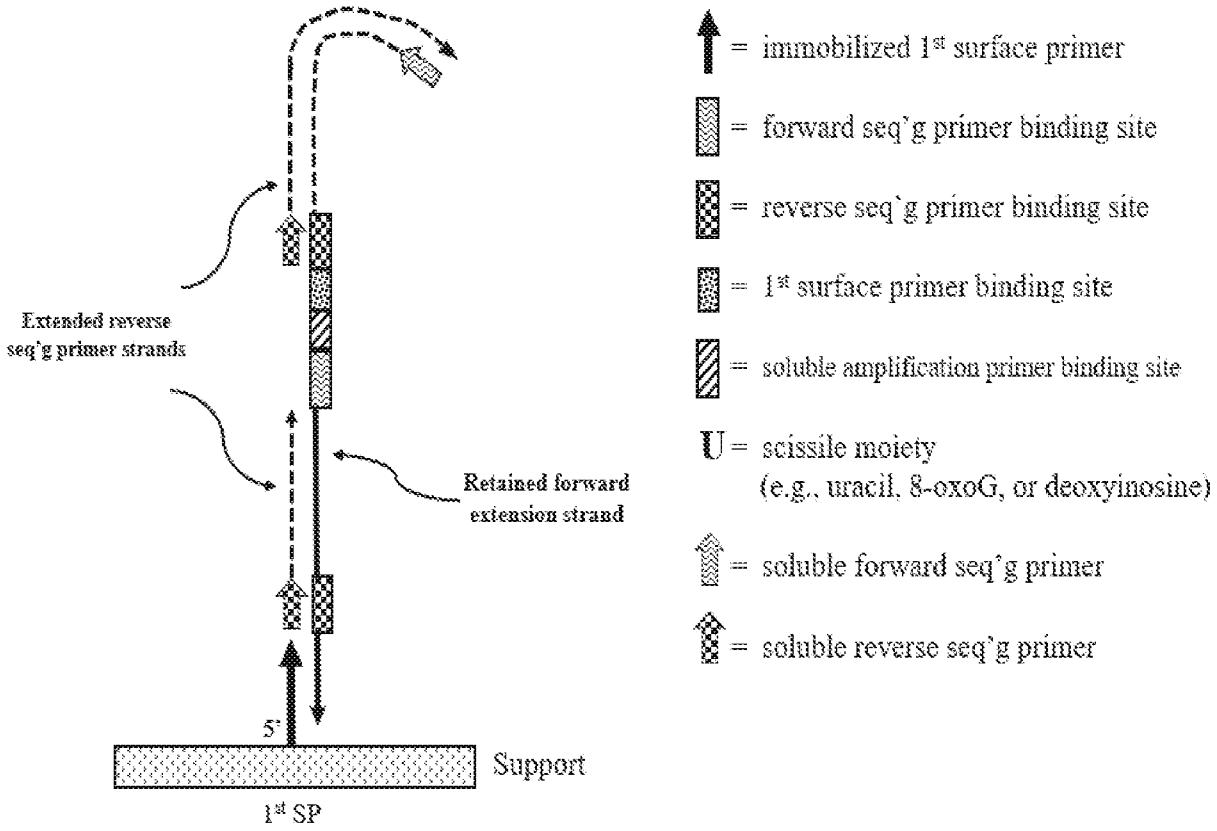

Extended reverse seq'g primer strands
Retained forward extension strand
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble forward seq'g primer
= soluble reverse seq'g primer

FIG. 10

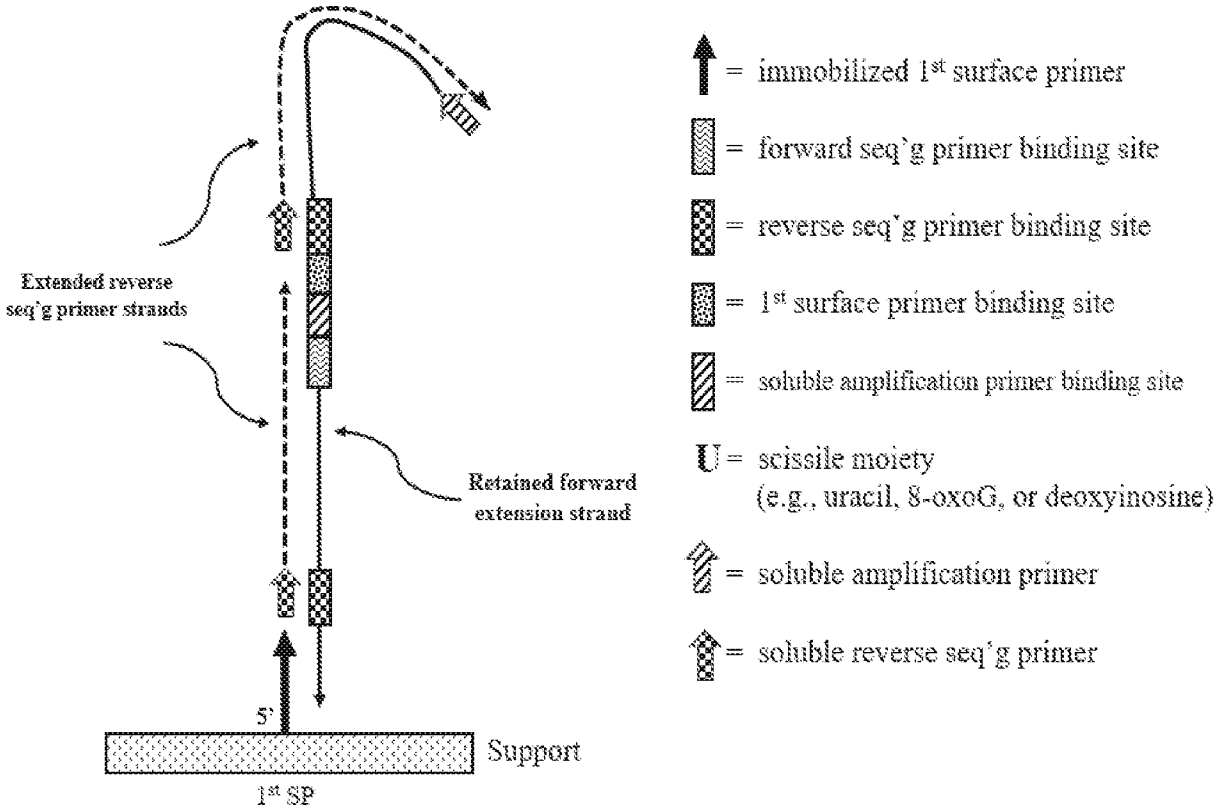

Extended reverse seq'g primer strands
Retained forward extension strand
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble amplification primer
= soluble reverse seq'g primer

FIG. 11

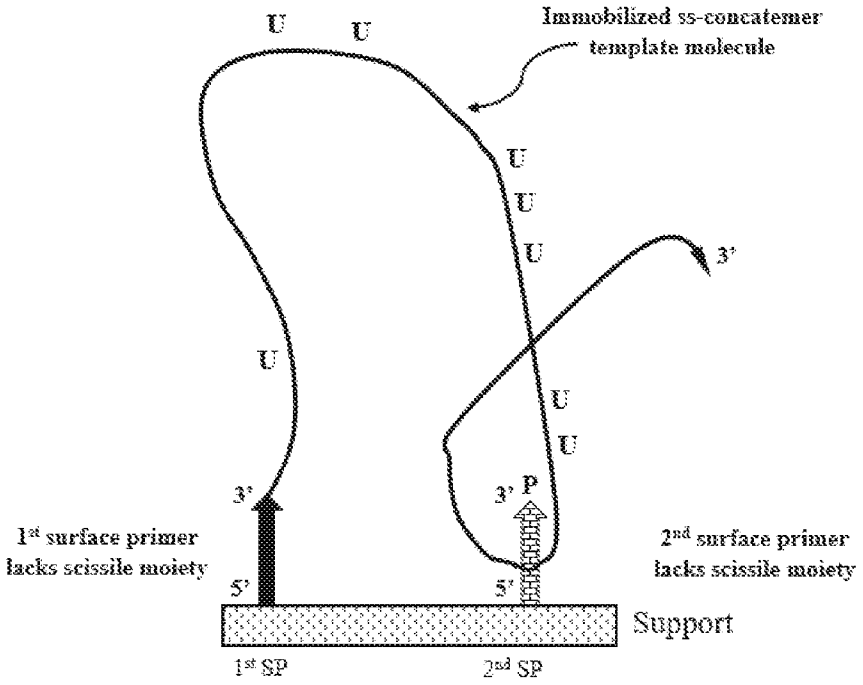

U
U
Immobilized ss-concatemer
template molecule
U
U
U
3'
U
U
U
3'
3' P
1st surface primer
lacks scissile moiety
2nd surface primer
lacks scissile moiety
5'
5'
Support
1st SP
2nd SP

FIG. 12

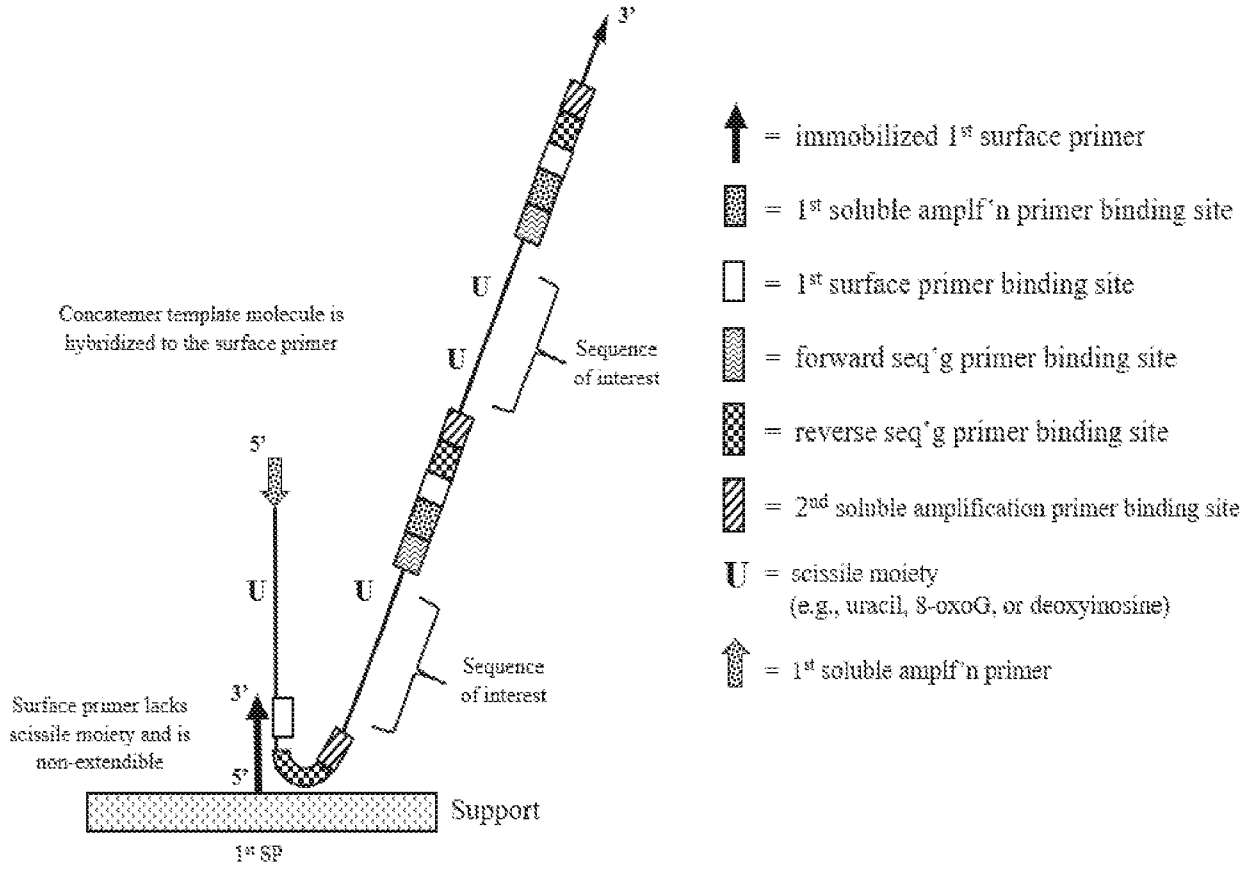

3'
U
U
Sequence of interest
Concatemer template molecule is hybridized to the surface primer
5'
U
U
Sequence of interest
3'
Surface primer lacks scissile moiety and is non-extendible
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= 1st soluble amplf'n primer

FIG. 13

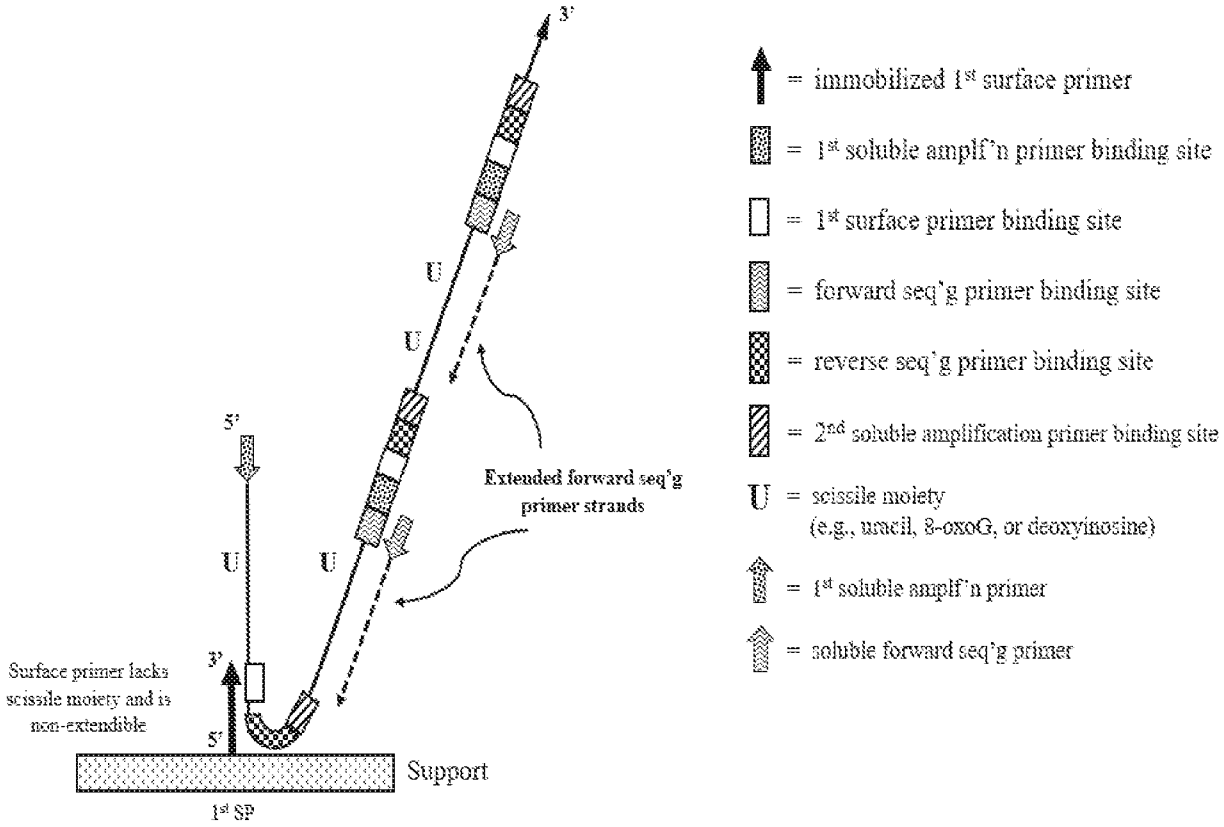

3'
U
U
5'
U
U
Extended forward seq'g primer strands
3'
Surface primer lacks scissile moiety and is non-extendible
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= 1st soluble amplf'n primer
= soluble forward seq'g primer

FIG. 14

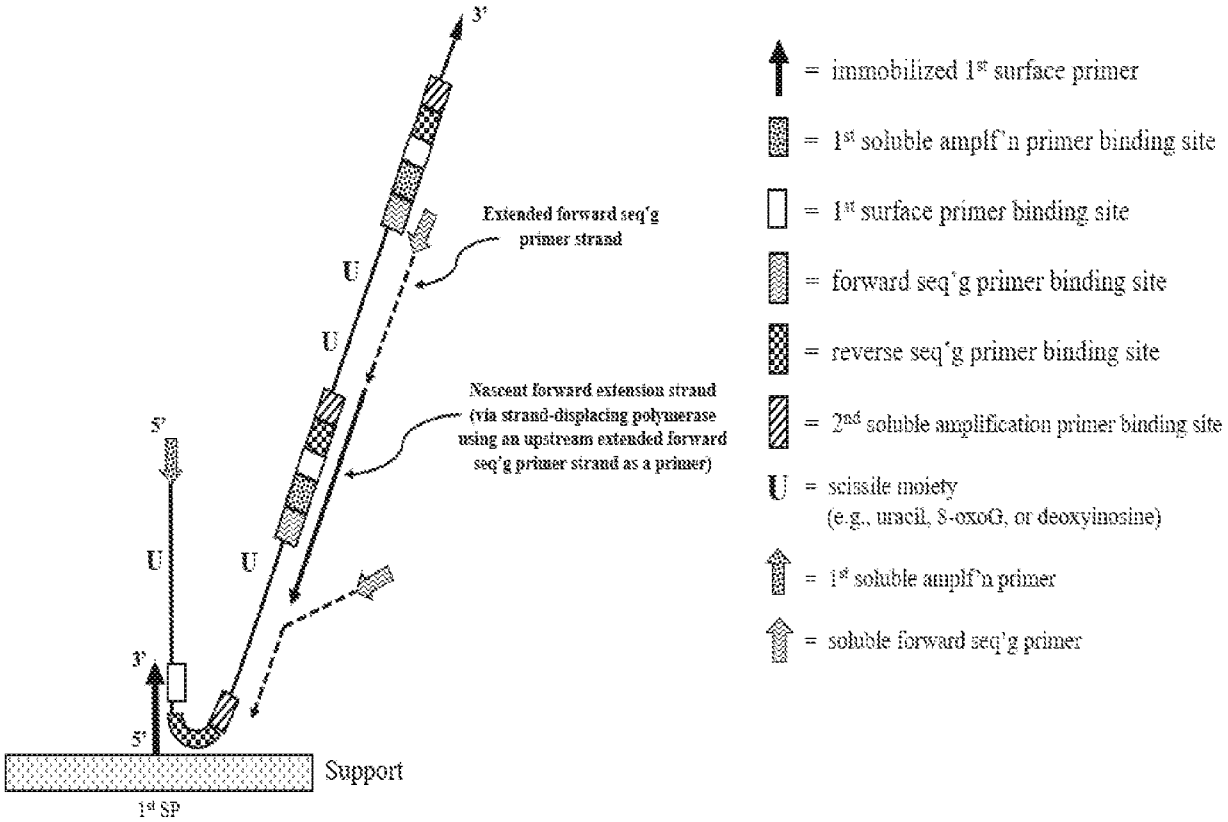

3'
Extended forward seq'g primer strand
U
U
Nascent forward extension strand (via strand-displacing polymerase using an upstream extended forward seq'g primer strand as a primer)
5'
U
U
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= 1st soluble amplf'n primer
= soluble forward seq'g primer

FIG. 15

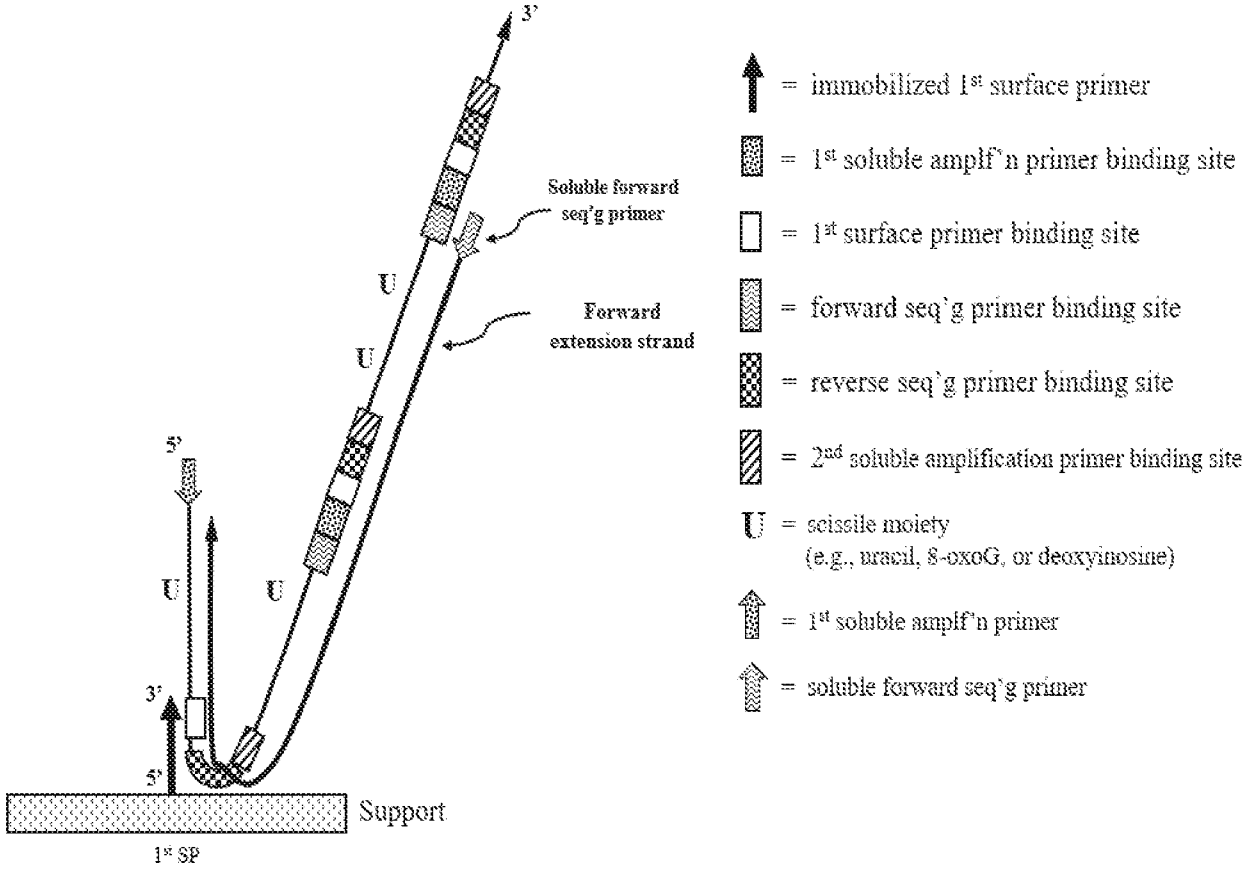

3'
Soluble forward seq'g primer
Forward extension strand
U
U
5'
U
U
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq`g primer binding site
= reverse seq`g primer binding site
= 2nd soluble amplification primer binding site
U = scissile moiety
(e.g., uracil, 8-oxoG, or deoxyinosine)
= 1st soluble amplf'n primer
= soluble forward seq`g primer

FIG. 16

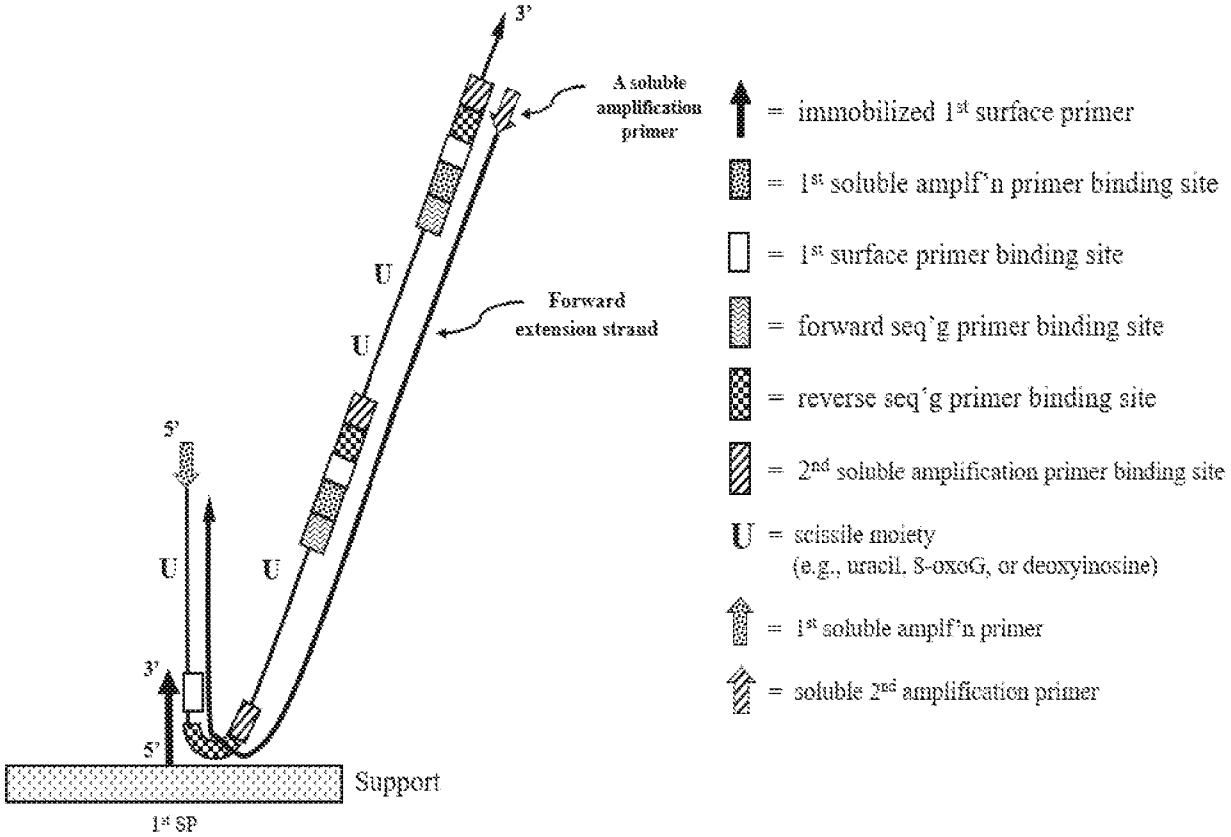

3'
A soluble amplification primer
Forward extension strand
U
U
U
U
5'
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
U = scissile moiety
(e.g., uracil, 8-oxoG, or deoxyinosine)
= 1st soluble amplf'n primer
= soluble 2nd amplification primer

FIG. 17

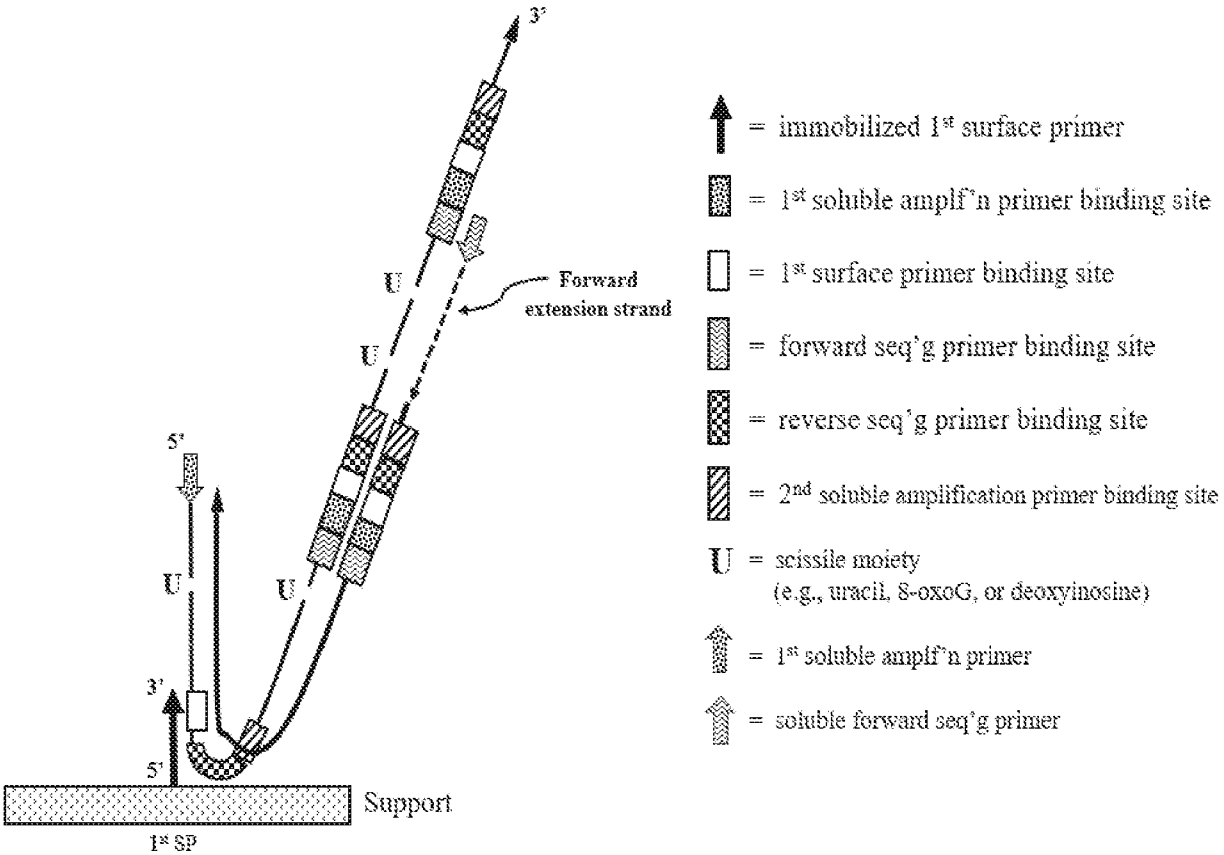

3'
U
U
Forward extension strand
5'
U
U
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
U = scissile moiety
(e.g., uracil, 8-oxoG, or deoxyinosine)
= 1st soluble amplf'n primer
= soluble forward seq'g primer

FIG. 18

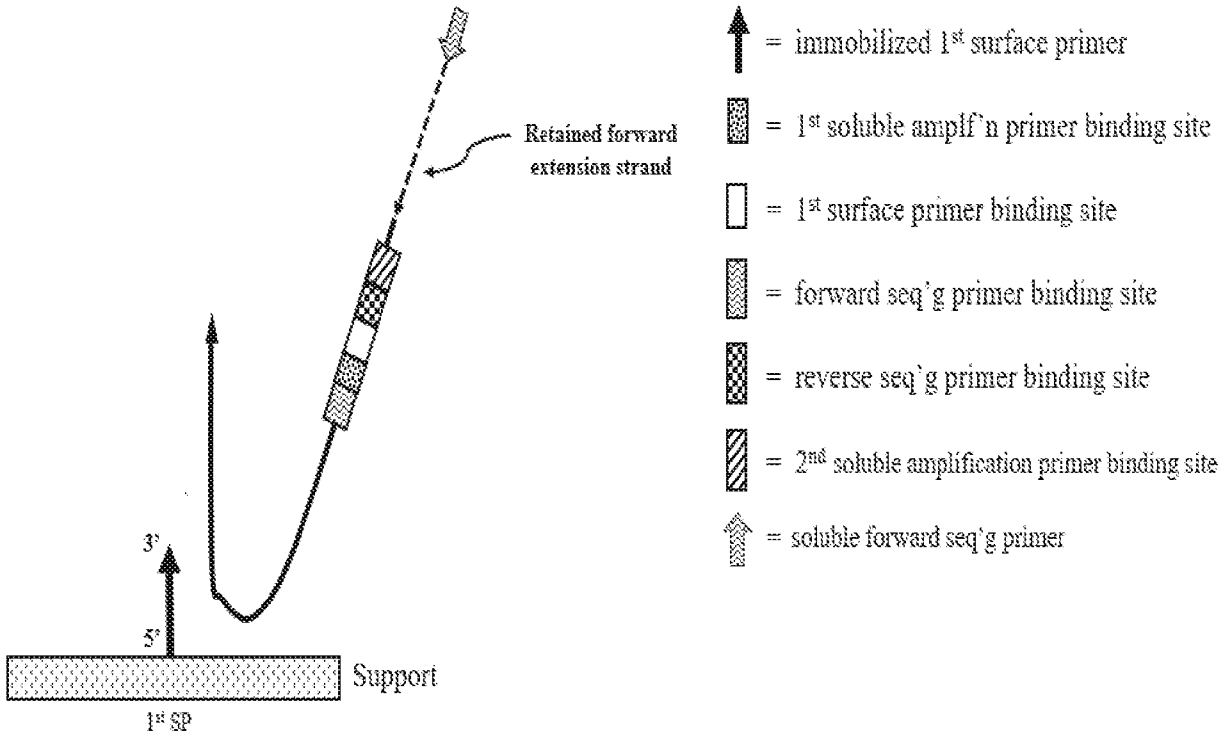

Retained forward extension strand
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf`n primer binding site
= 1st surface primer binding site
= forward seq`g primer binding site
= reverse seq`g primer binding site
= 2nd soluble amplification primer binding site
= soluble forward seq`g primer

FIG. 19

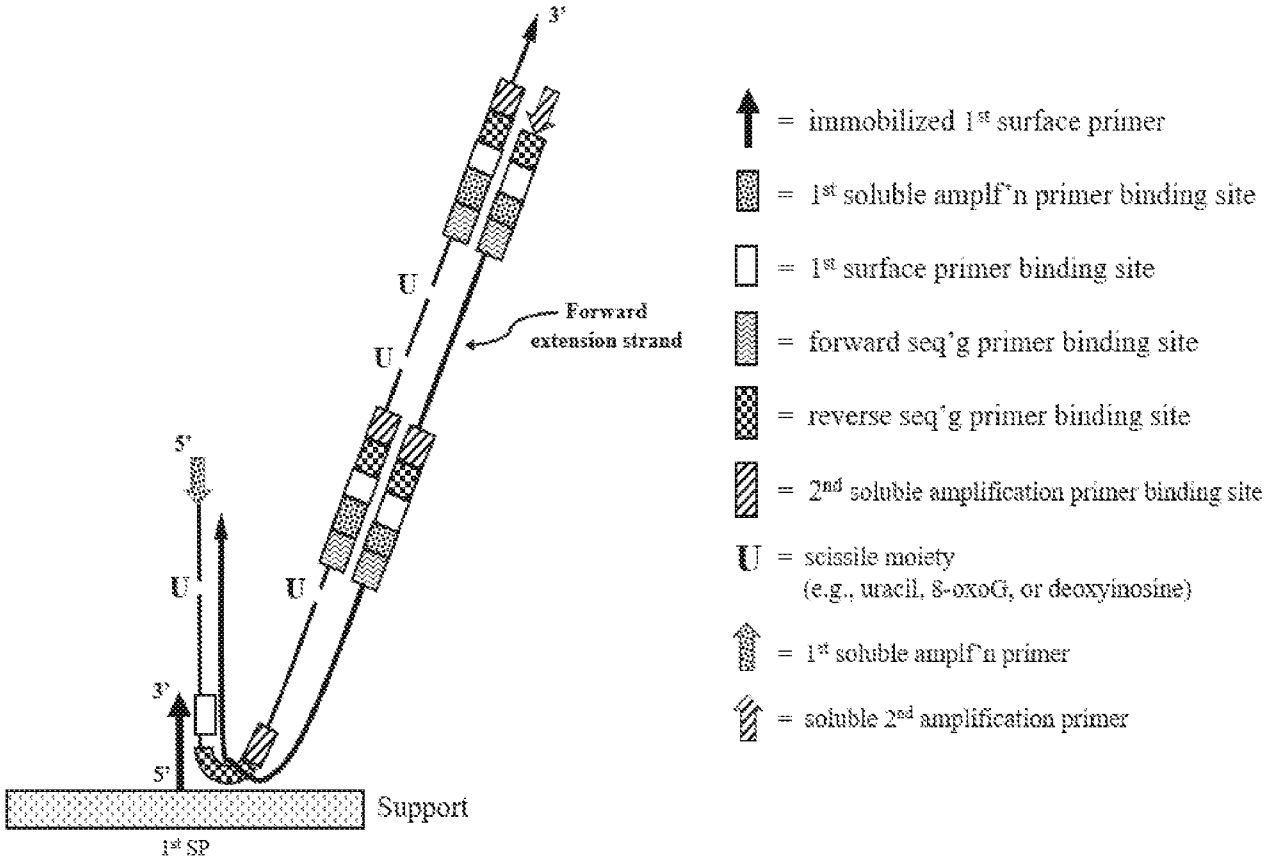

3'
U
U
Forward extension strand
5'
U
U
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= 1st soluble amplf'n primer
= soluble 2nd amplification primer

FIG. 20

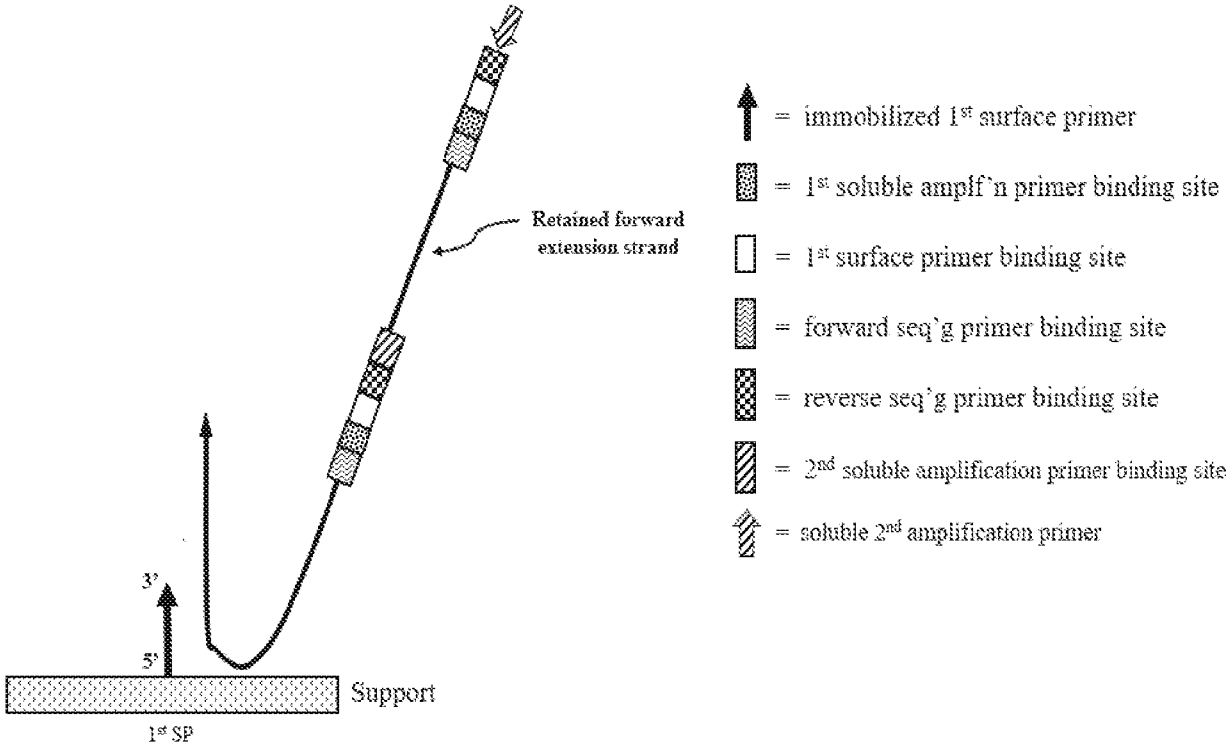

Retained forward extension strand
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
= soluble 2nd amplification primer

FIG. 21

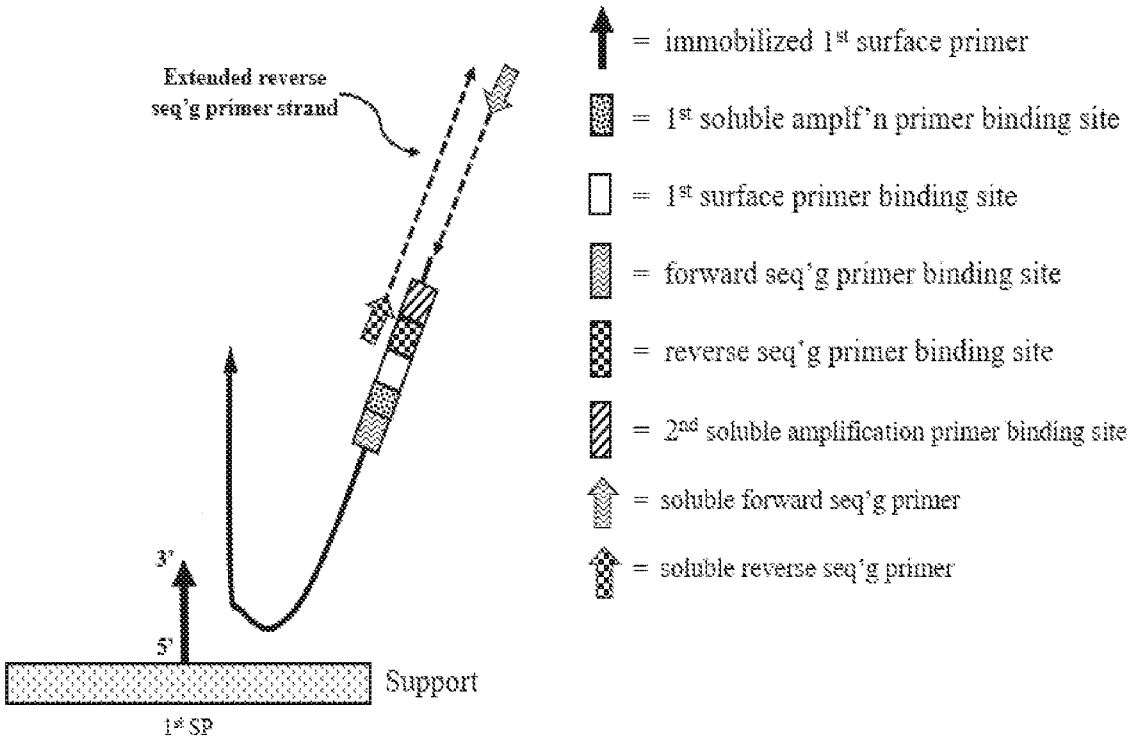

Extended reverse seq'g primer strand
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
= soluble forward seq'g primer
= soluble reverse seq'g primer

FIG. 22

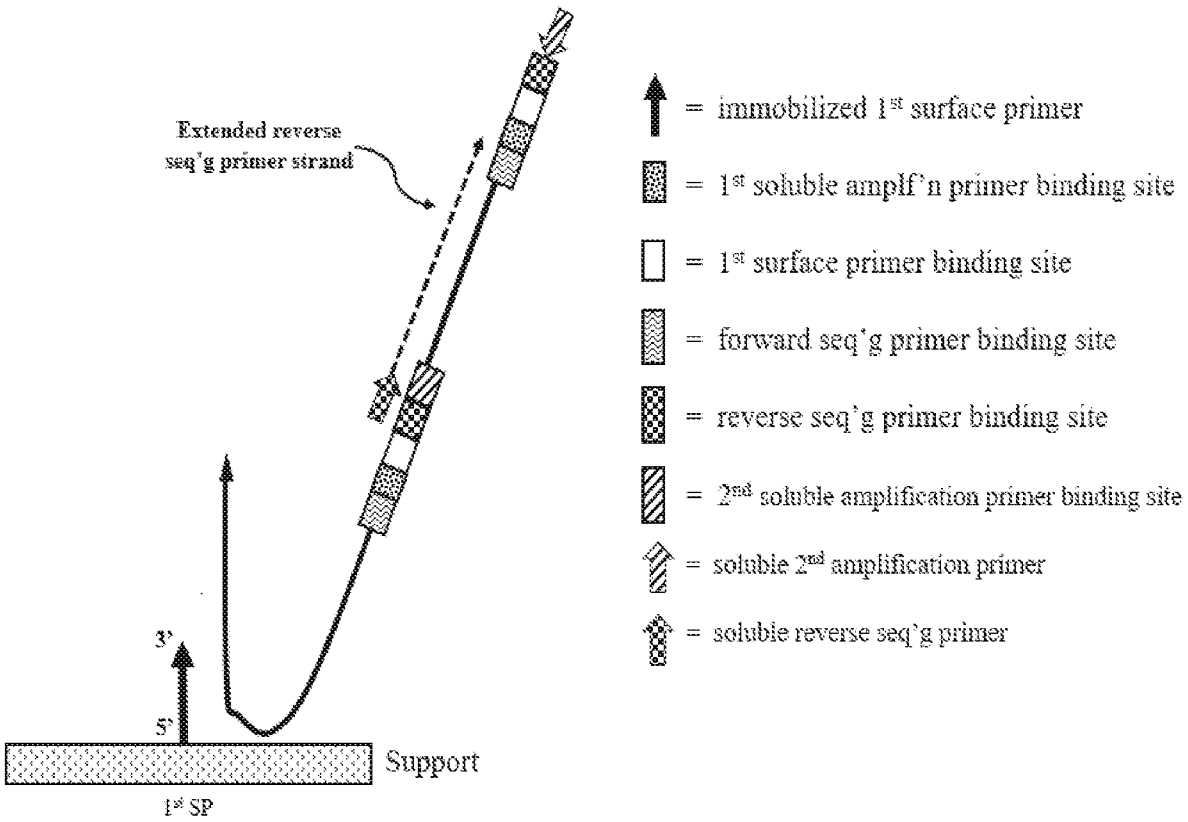

Extended reverse seq'g primer strand
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
= soluble 2nd amplification primer
= soluble reverse seq'g primer

FIG. 23

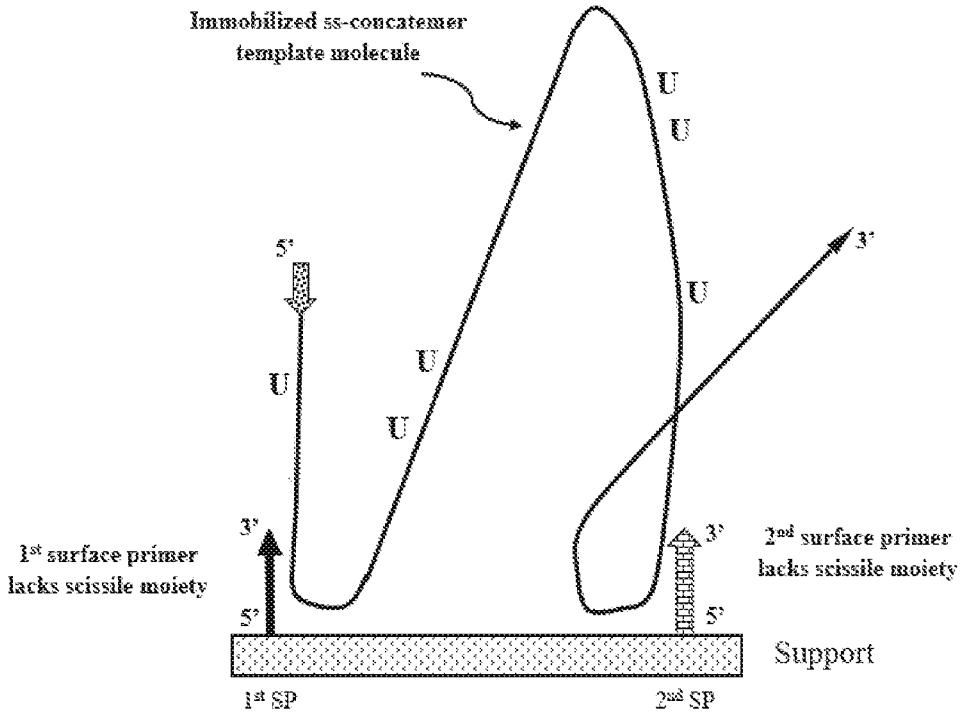

Immobilized ss-concatemer template molecule
U
U
5'
3'
U
U
U
U
3'
1st surface primer lacks scissile moiety
3'
2nd surface primer lacks scissile moiety
5'
5'
Support
1st SP
2nd SP

FIG. 24

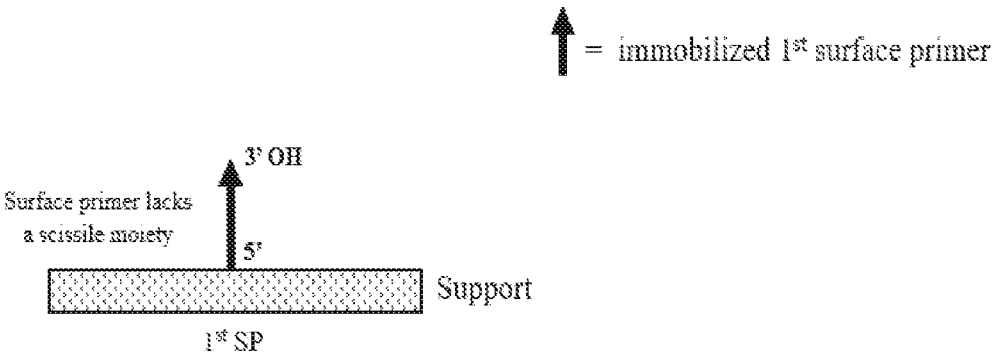

= immobilized 1st surface primer
3' OH
Surface primer lacks
a scissile moiety
5'
Support
1st SP

FIG. 25

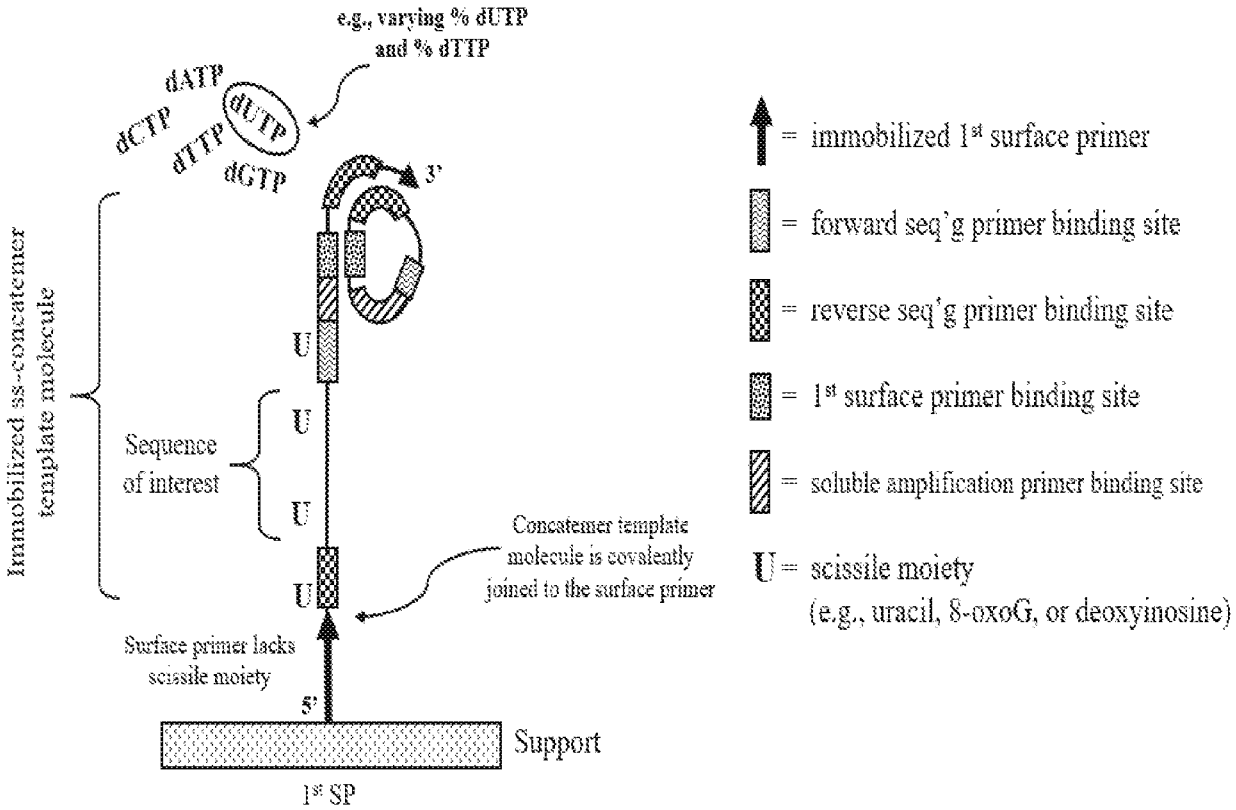

e.g., varying % dUTP
and % dTTP
dATP
dCTP
dTTP
dUTP
dGTP
3'
Immobilized ss-concatemer
template molecule
U
U
U
U
Sequence
of interest
Concatemer template
molecule is covalently
joined to the surface primer
Surface primer lacks
scissile moiety
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety
(e.g., uracil, 8-oxoG, or deoxyinosine)

FIG. 26

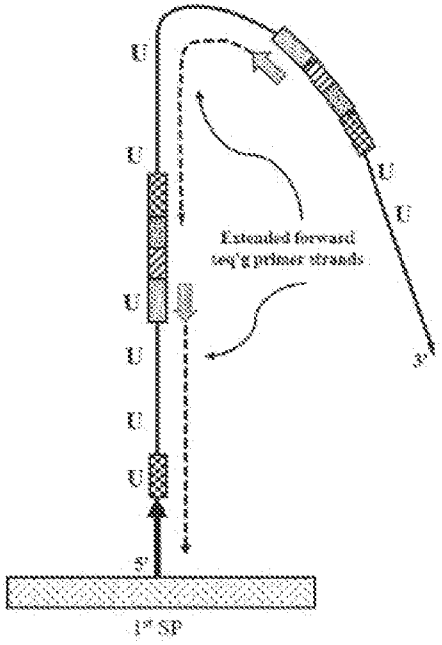


FIG. 27

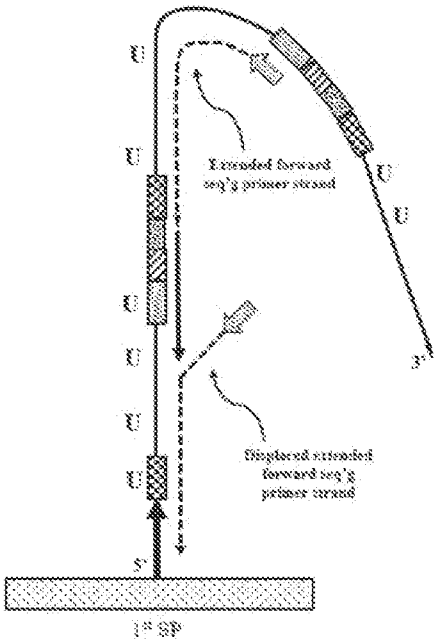


FIG. 28

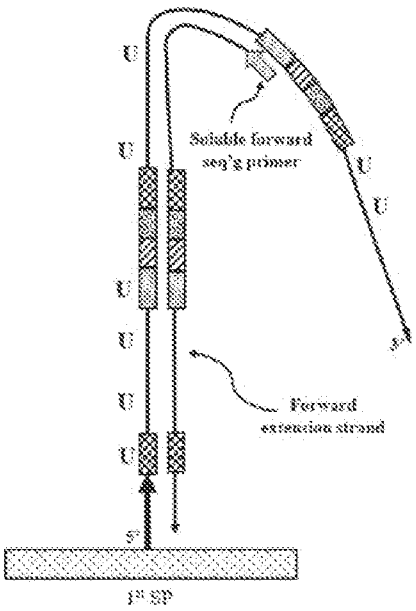


FIG. 29

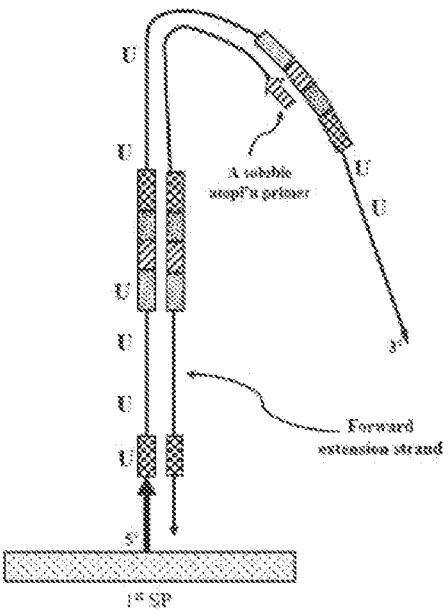


FIG. 30

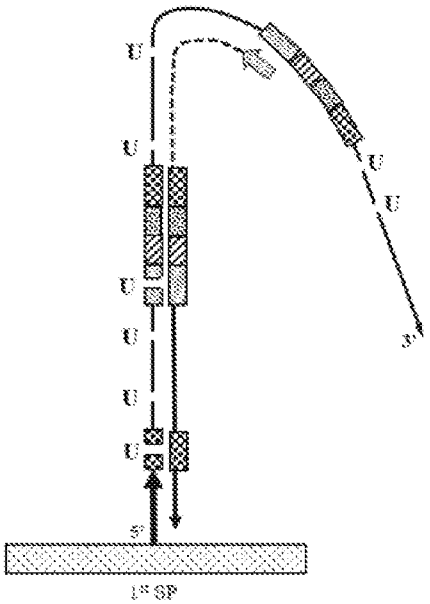


FIG. 31

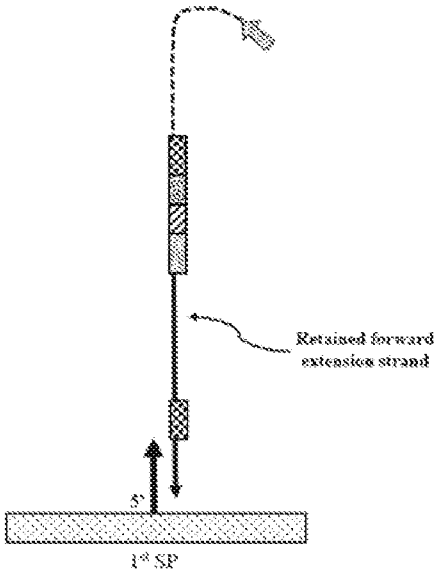


FIG. 32

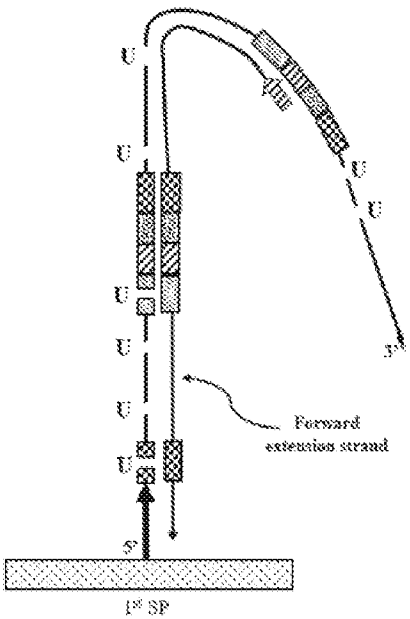


FIG. 33

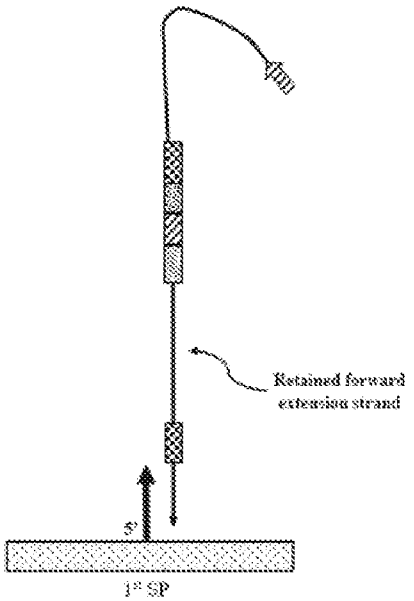


FIG. 34

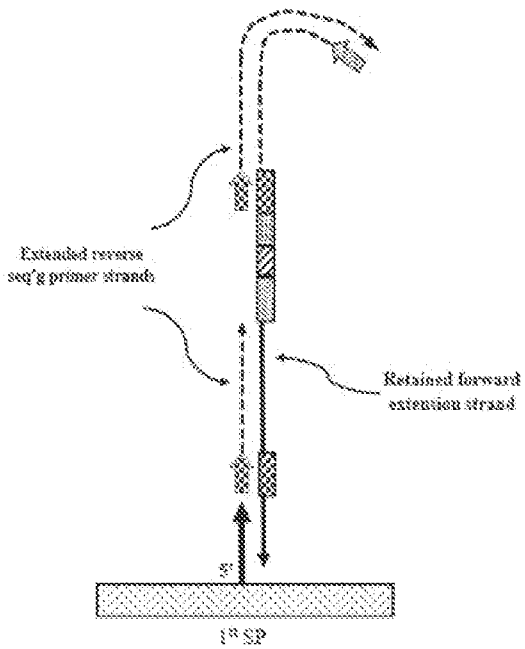


FIG. 35

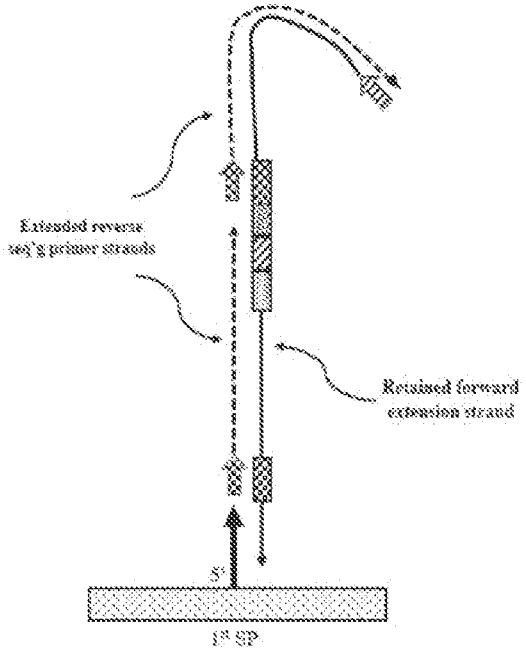


FIG. 36

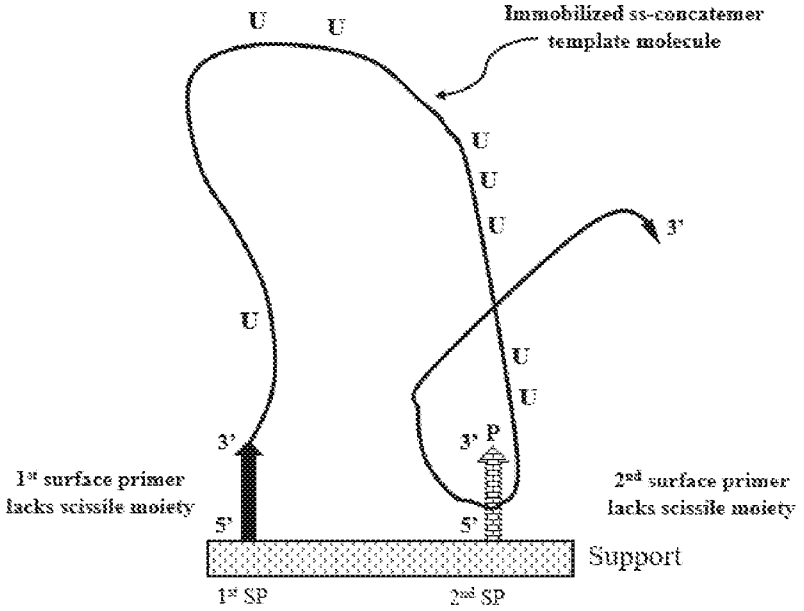


FIG. 37

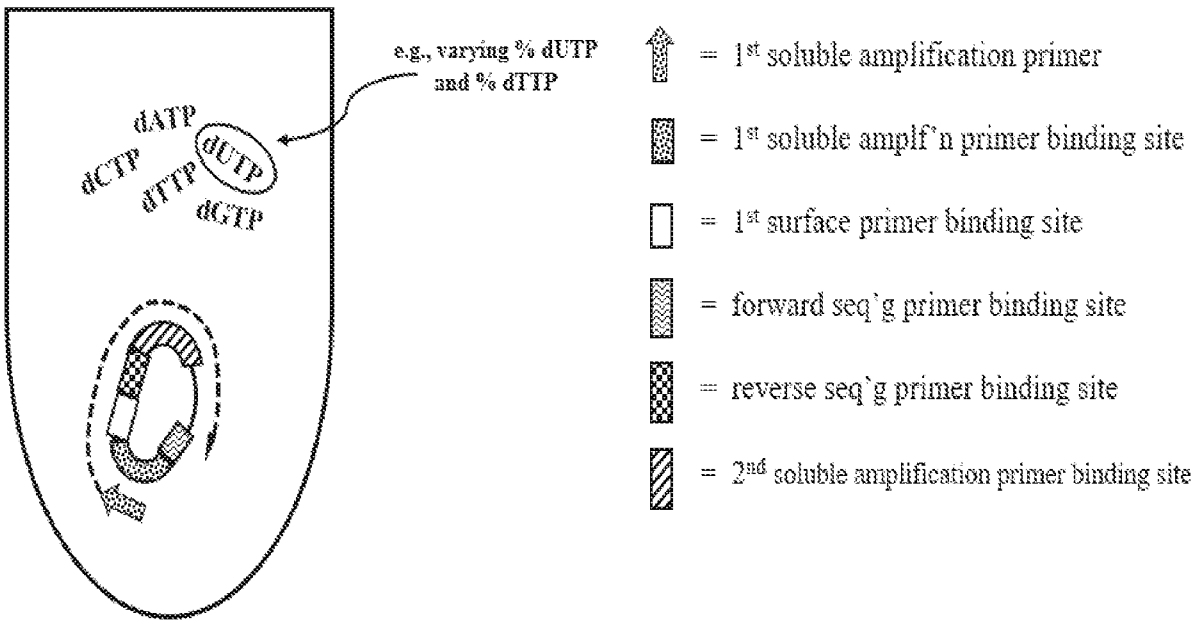

e.g., varying % dUTP
and % dTTP
dATP
dCTP
dTTP
dUTP
dGTP
= 1st soluble amplification primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site

FIG. 38

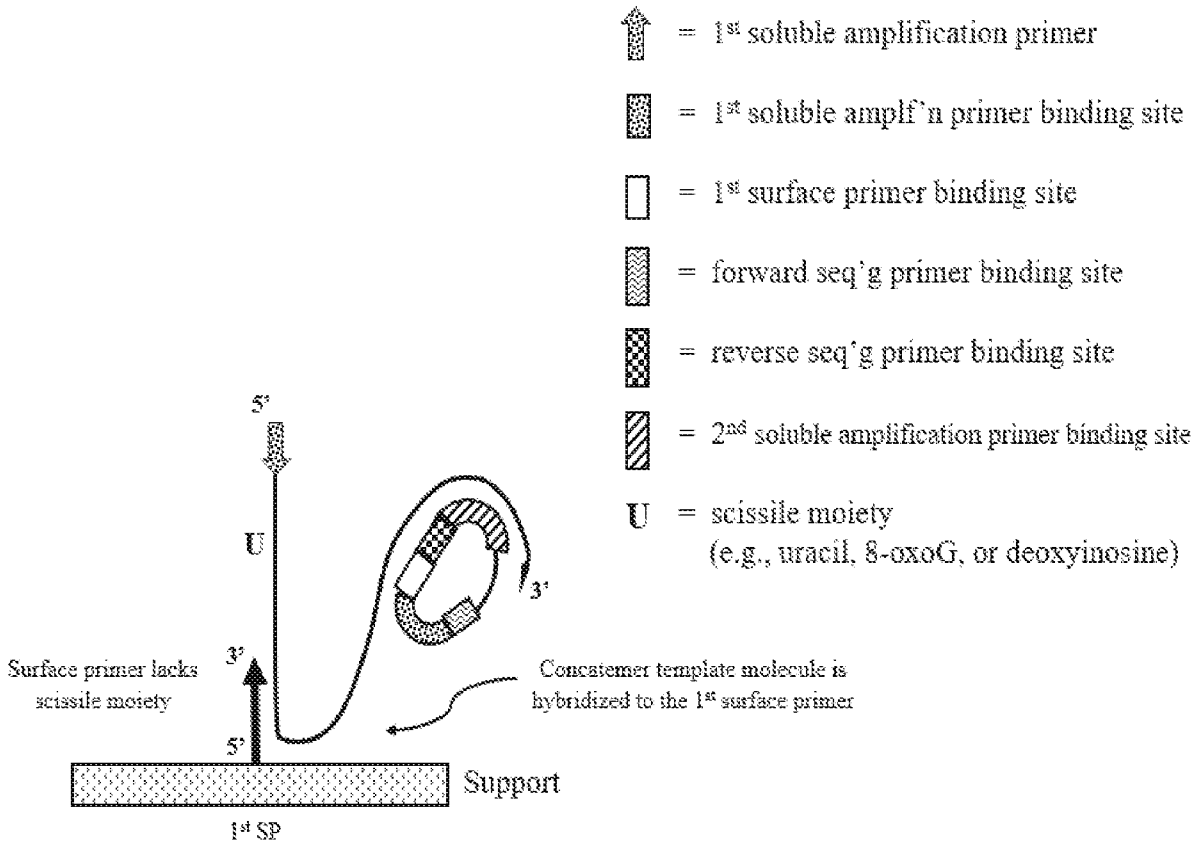

= 1st soluble amplification primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
U = scissile moiety
(e.g., uracil, 8-oxoG, or deoxyinosine)
5'
U
3'
3'
Surface primer lacks scissile moiety
Concatemer template molecule is hybridized to the 1st surface primer
5'
Support
1st SP

FIG. 39

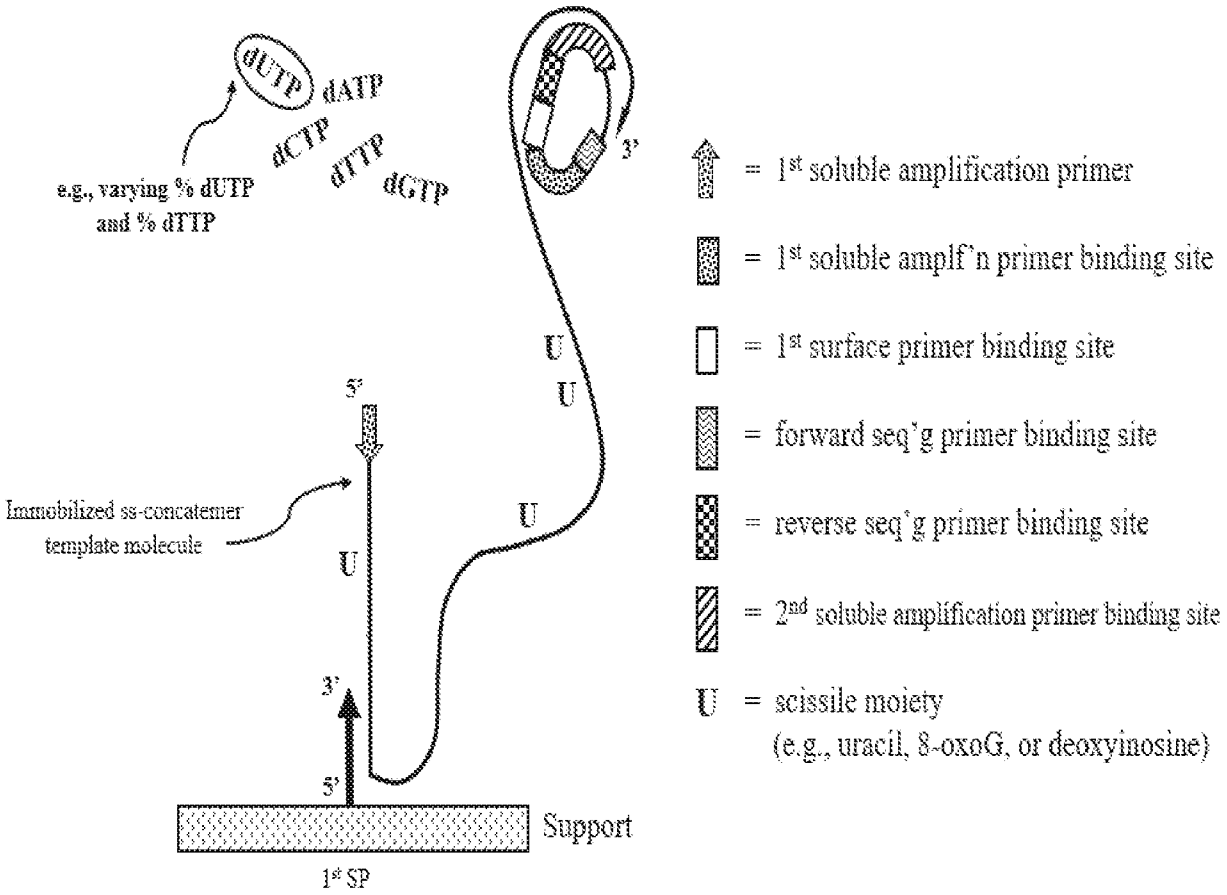

dUTP
dATP
dCTP
dTTP
dGTP
e.g., varying % dUTP
and % dTTP
3'
U
U
5'
Immobilized ss-concatemer
template molecule
U
U
3'
5'
Support
1st SP
= 1st soluble amplification primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
U = scissile moiety
(e.g., uracil, 8-oxoG, or deoxyinosine)

FIG. 40

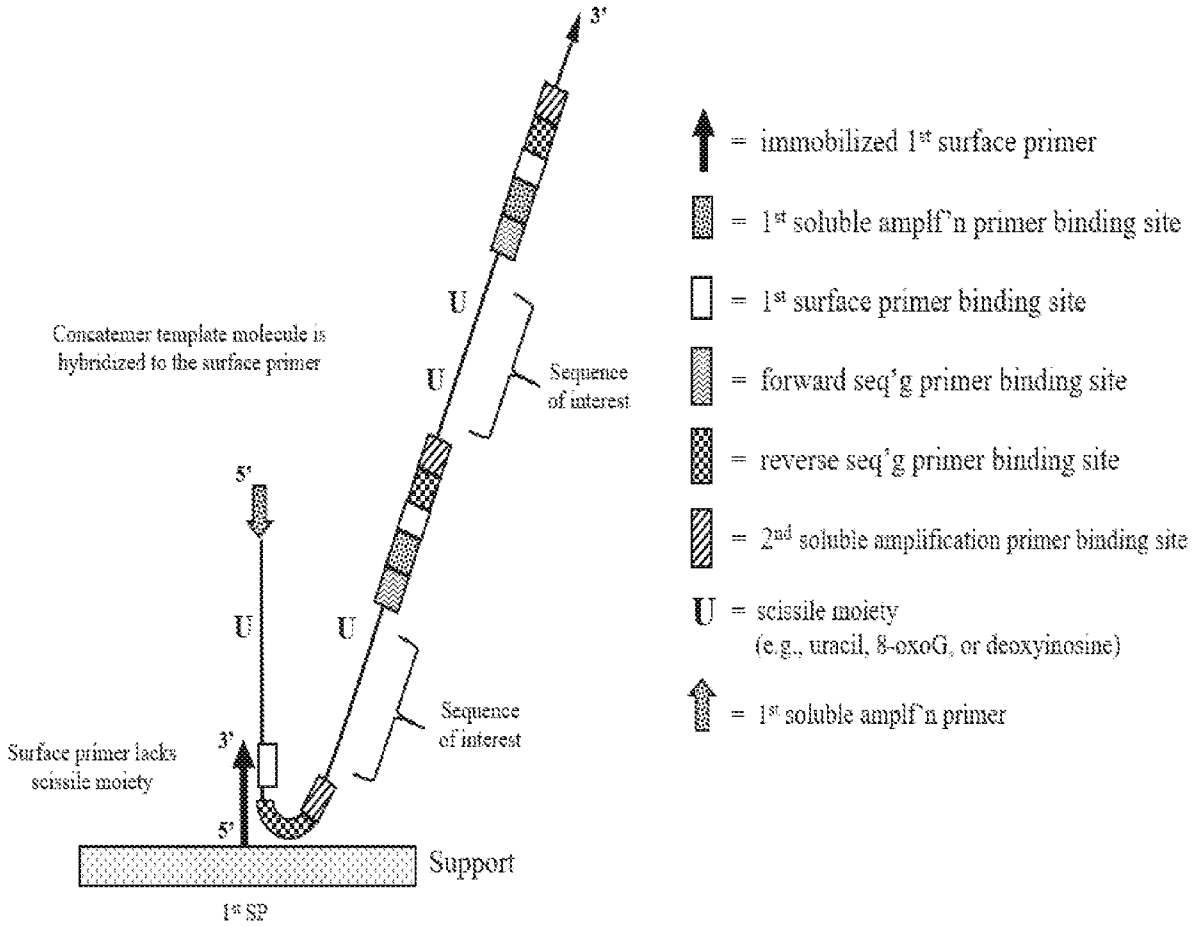

3'
U
U
Concatemer template molecule is hybridized to the surface primer
Sequence of interest
5'
U
U
Sequence of interest
3'
Surface primer lacks scissile moiety
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= 1st soluble amplf'n primer

FIG. 41

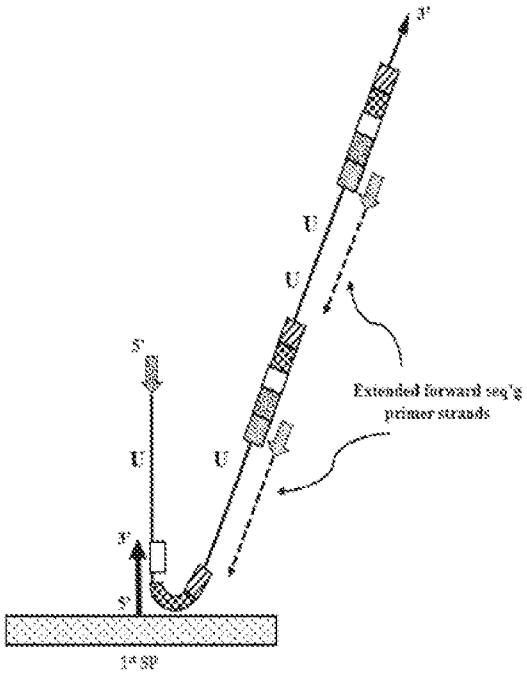


FIG. 42

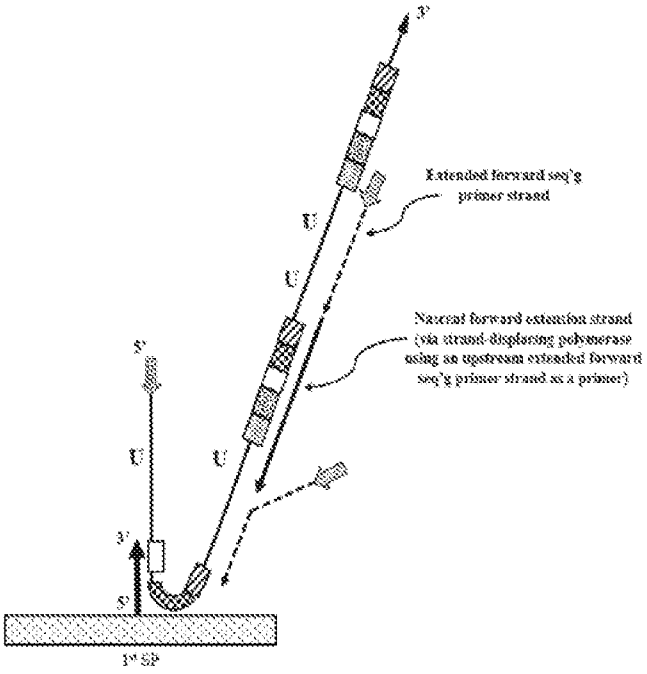


FIG. 43

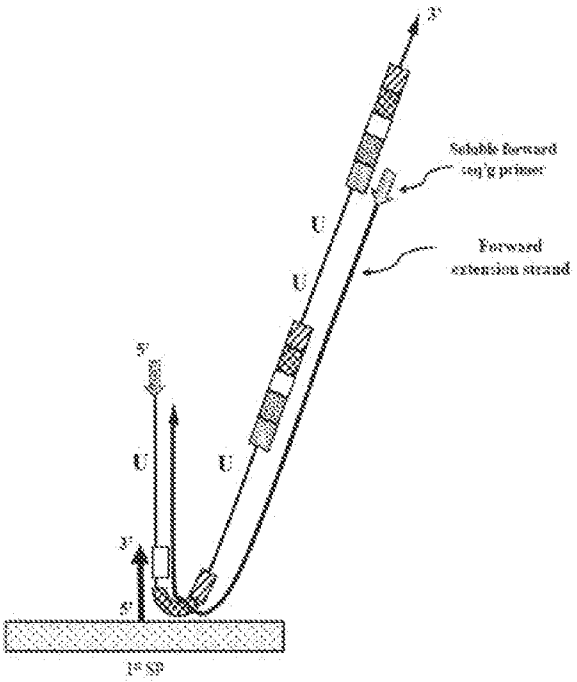


FIG. 44

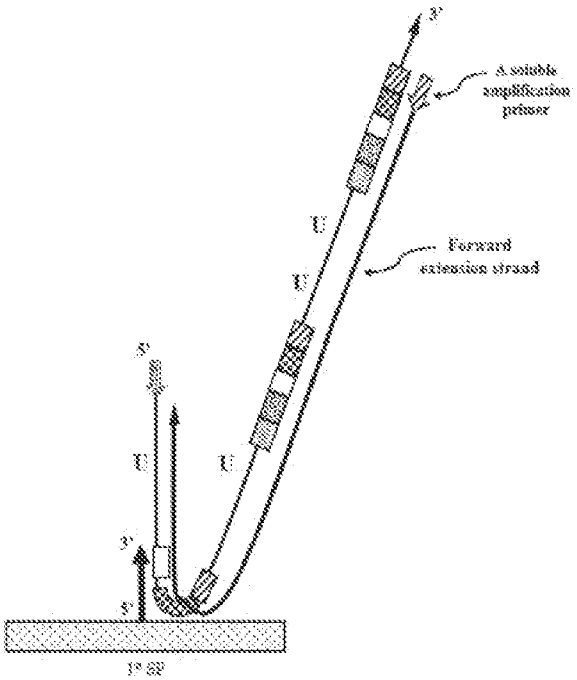


FIG. 45

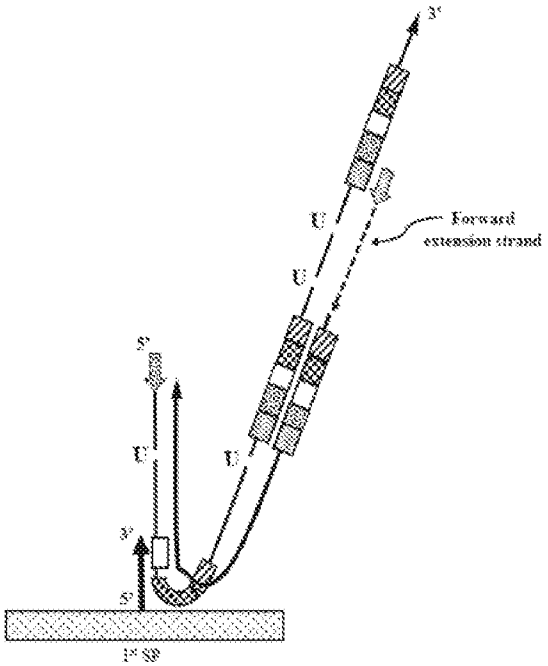


FIG. 46

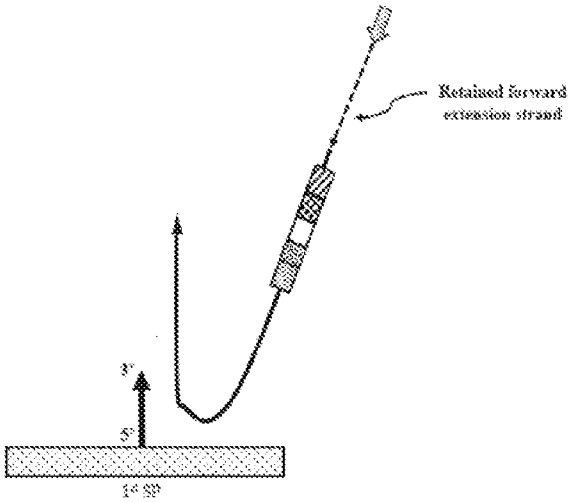


FIG. 47

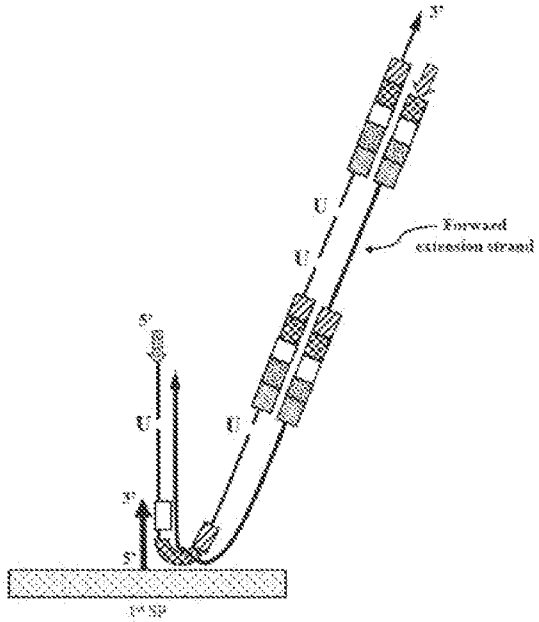


FIG. 48

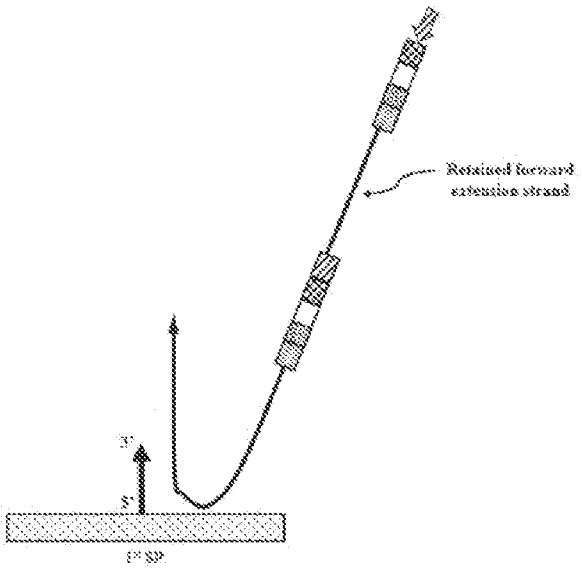


FIG. 49

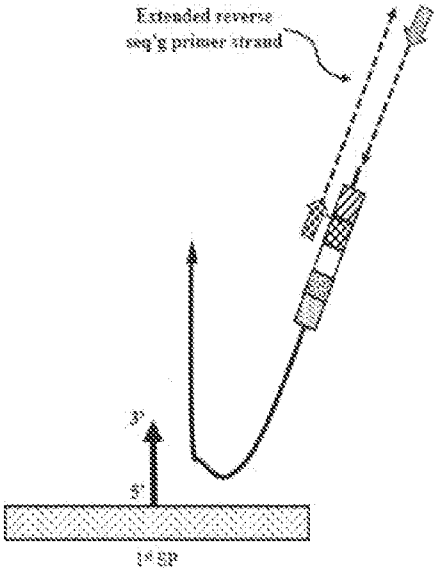


FIG. 50

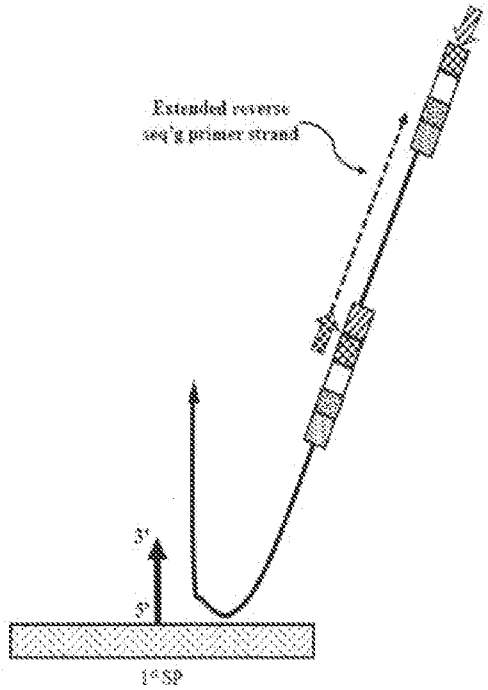


FIG. 51

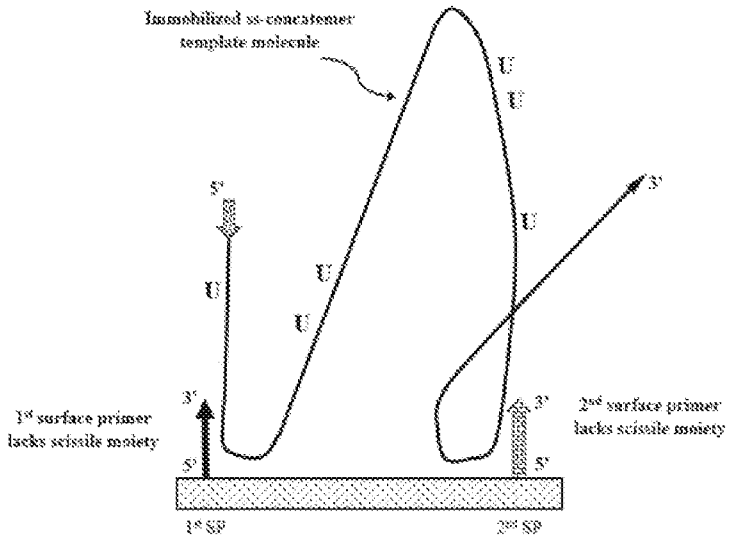


FIG. 52

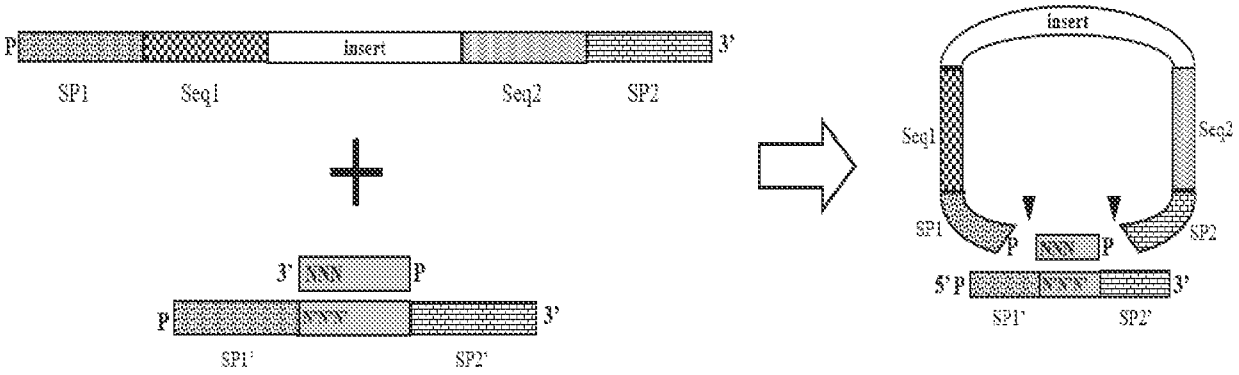

P
insert
3'
SP1
Seq1
Seq2
SP2
3'
P
P
3'
SP1'
SP2'
insert
Seq1
Seq2
SP1
P
P
SP2
5'P
3'
SP1'
SP2'

FIG. 53

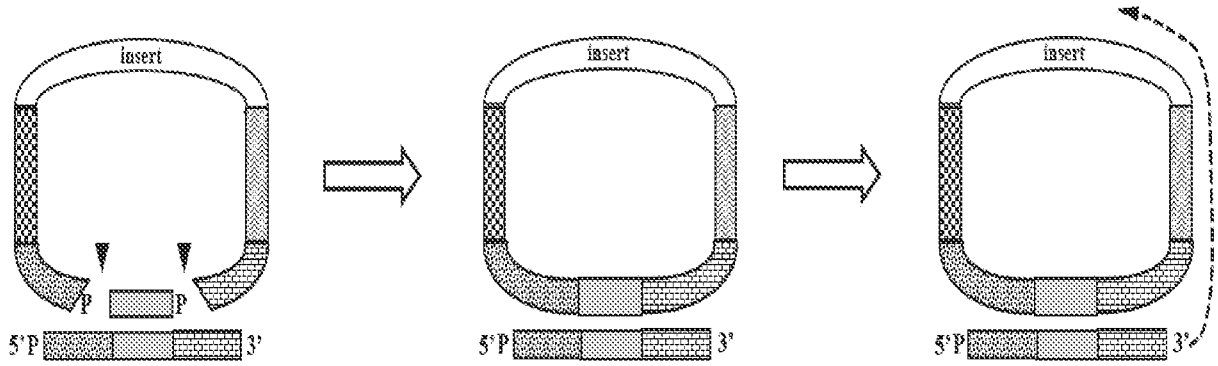

insert
P
P
5'P
3'
insert
5'P
3'
insert
5'P
3'

FIG. 54

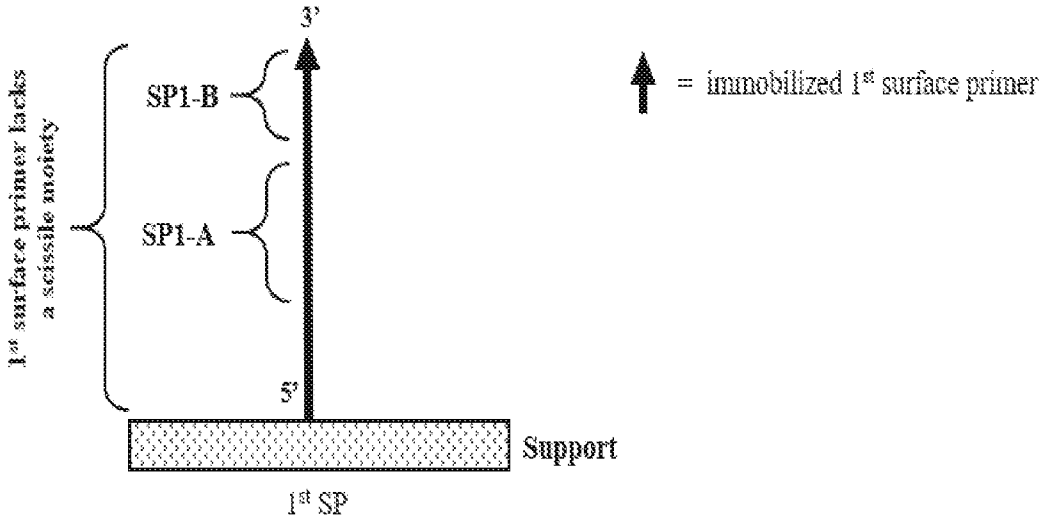

3'
SP1-B
SP1-A
1st surface primer lacks a scissile moiety
5'
Support
1st SP
= immobilized 1st surface primer

FIG. 55

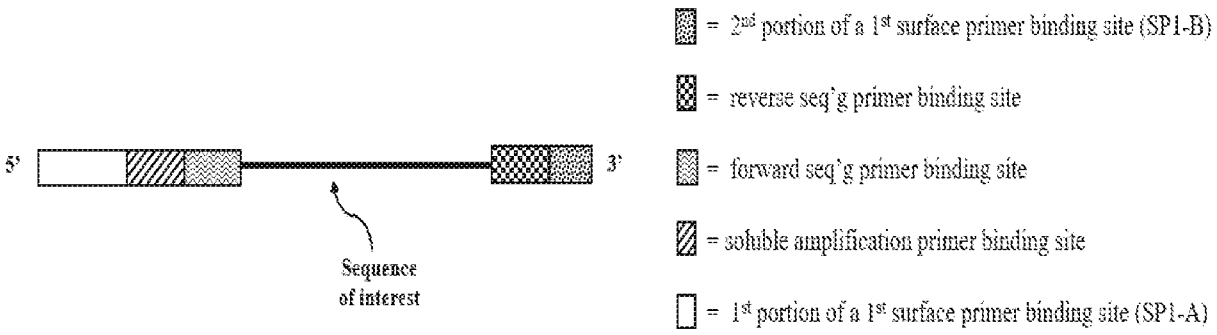

5'
3'
Sequence of interest
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)

FIG. 56

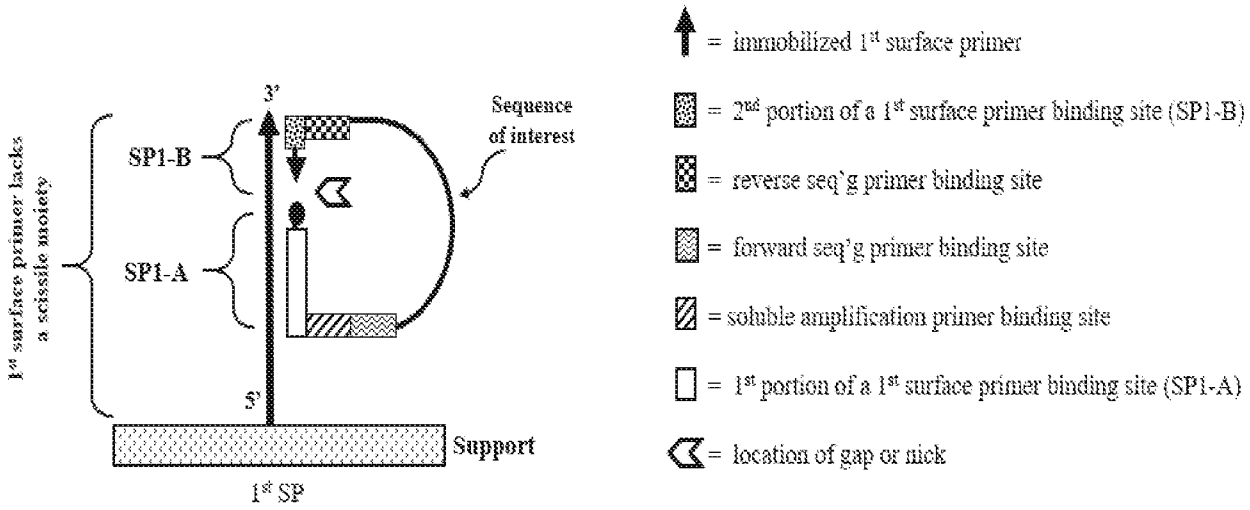

1st surface primer lacks a scissile moiety
SP1-B
SP1-A
3'
5'
Sequence of interest
Support
1st SP
= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)
= location of gap or nick

FIG. 57

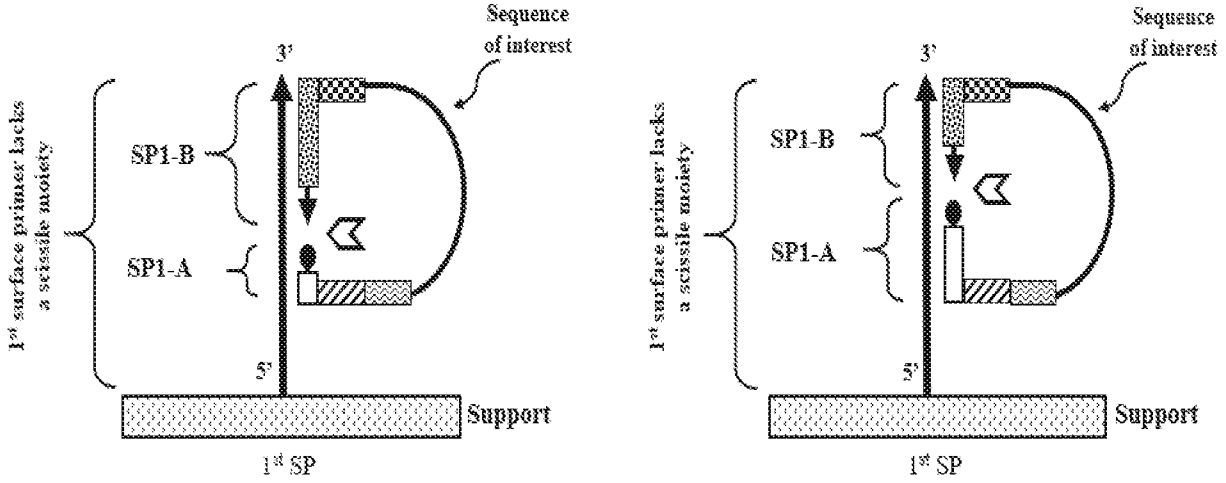

1st surface primer lacks a scissile moiety
SP1-B
SP1-A
3'
5'
Sequence of interest
Support
1st SP
1st surface primer lacks a scissile moiety
SP1-B
SP1-A
3'
5'
Sequence of interest
Support
1st SP

FIG. 58

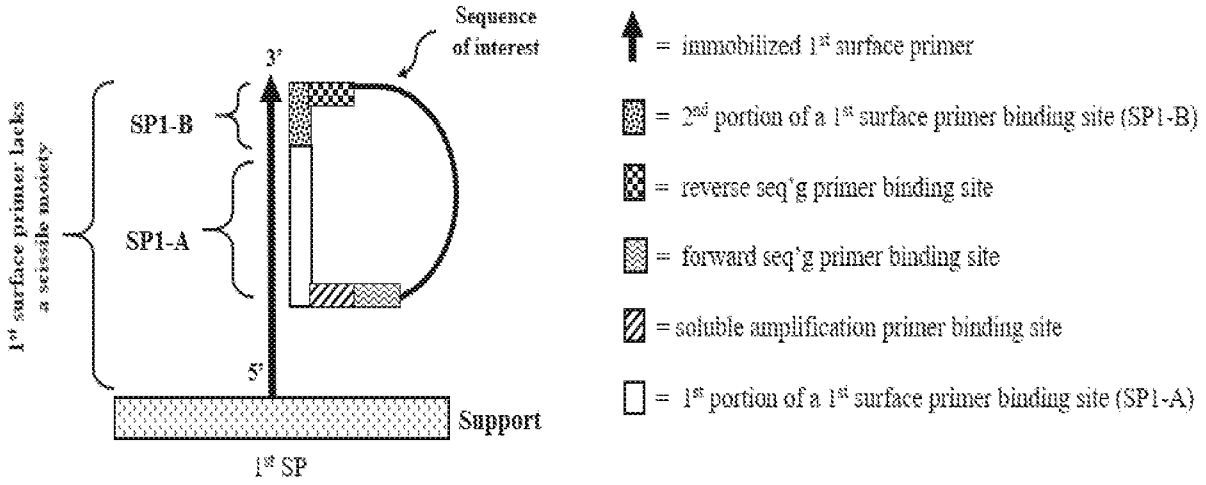

Sequence of interest
3'
SP1-B
SP1-A
1st surface primer lacks a scissile moiety
5'
Support
1st SP
= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)

FIG. 59

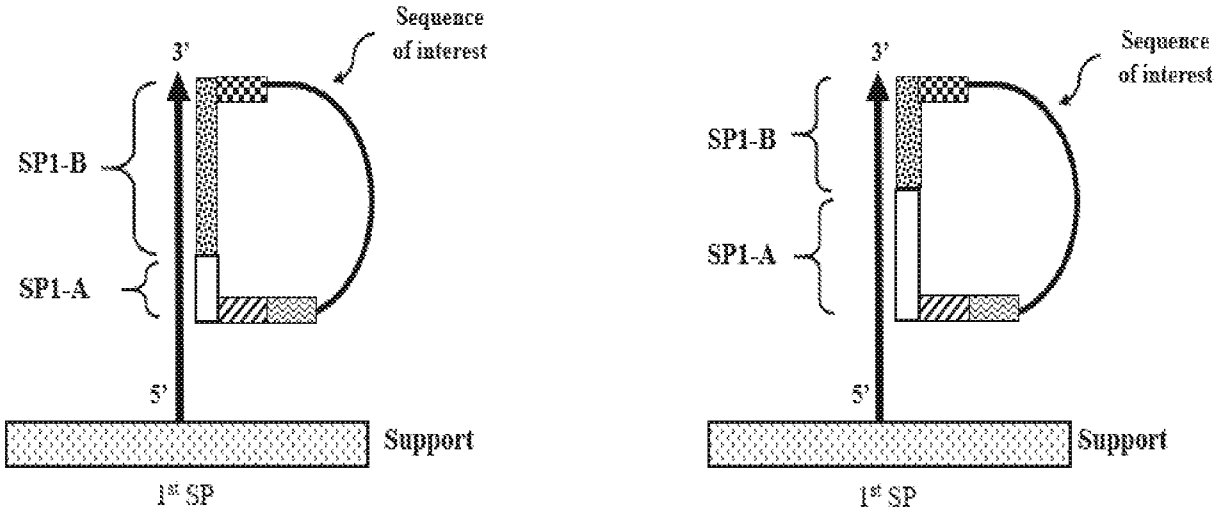

Sequence of interest
3'
SP1-B
SP1-A
5'
Support
1st SP
Sequence of interest
3'
SP1-B
SP1-A
5'
Support
1st SP

FIG. 60

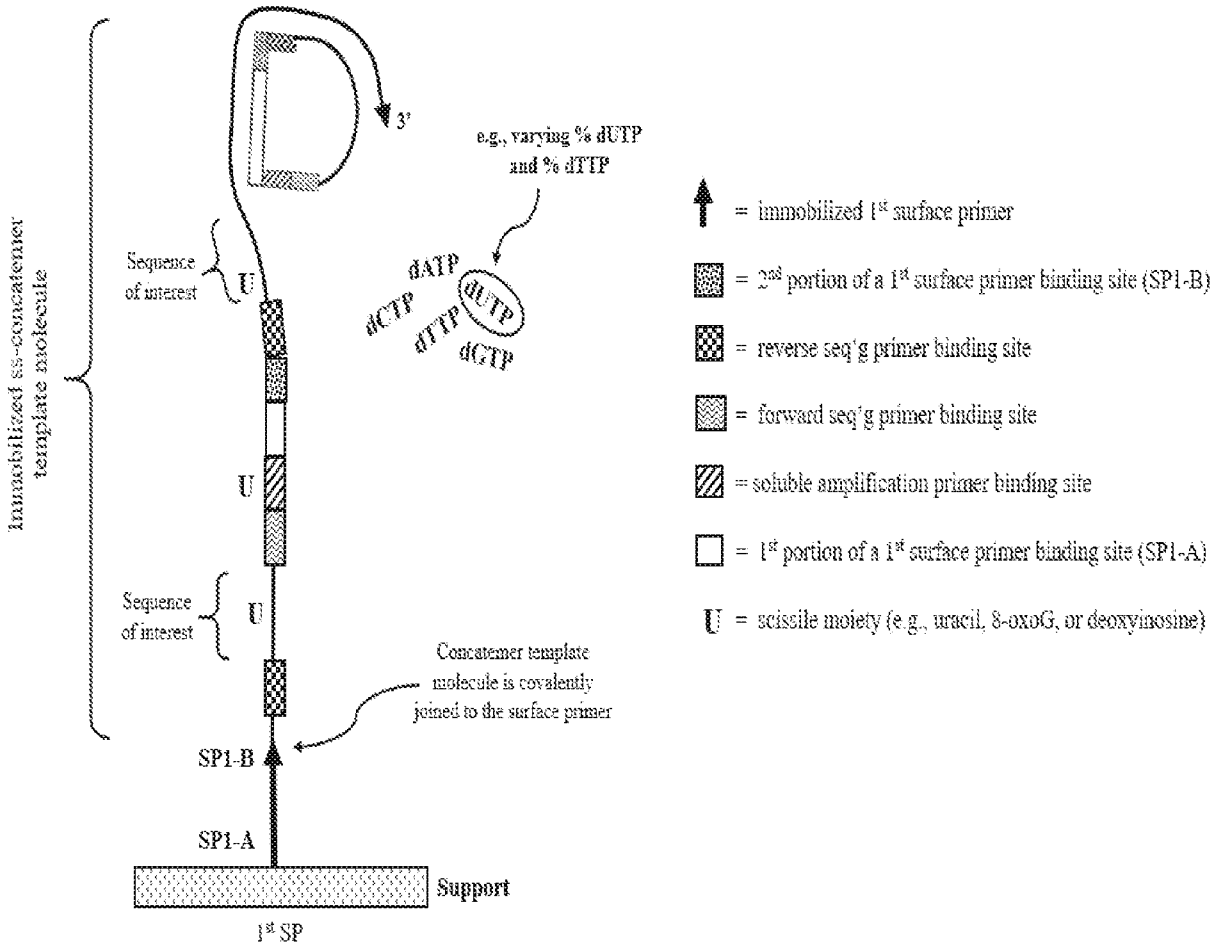

Immobilized ss-concatemer template molecule
3'
e.g., varying % dUTP and % dTTP
dATP
dCTP
dTTP
dUTP
dGTP
Sequence of interest
U
U
Sequence of interest
U
Concatemer template molecule is covalently joined to the surface primer
SP1-B
SP1-A
Support
1st SP
= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)

FIG. 61

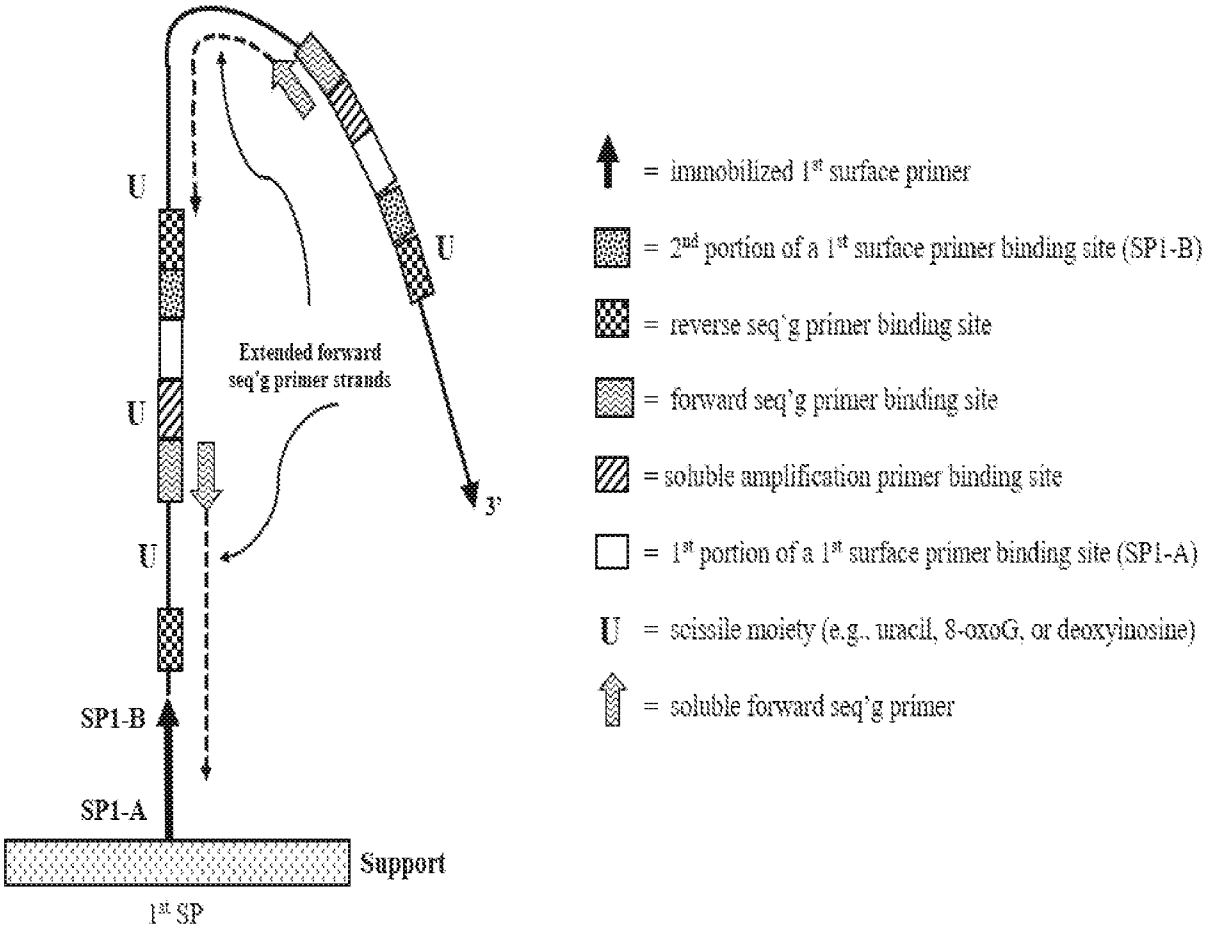

U
U
U
U
Extended forward seq'g primer strands
3'
SP1-B
SP1-A
Support
1st SP
= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble forward seq'g primer

FIG. 62

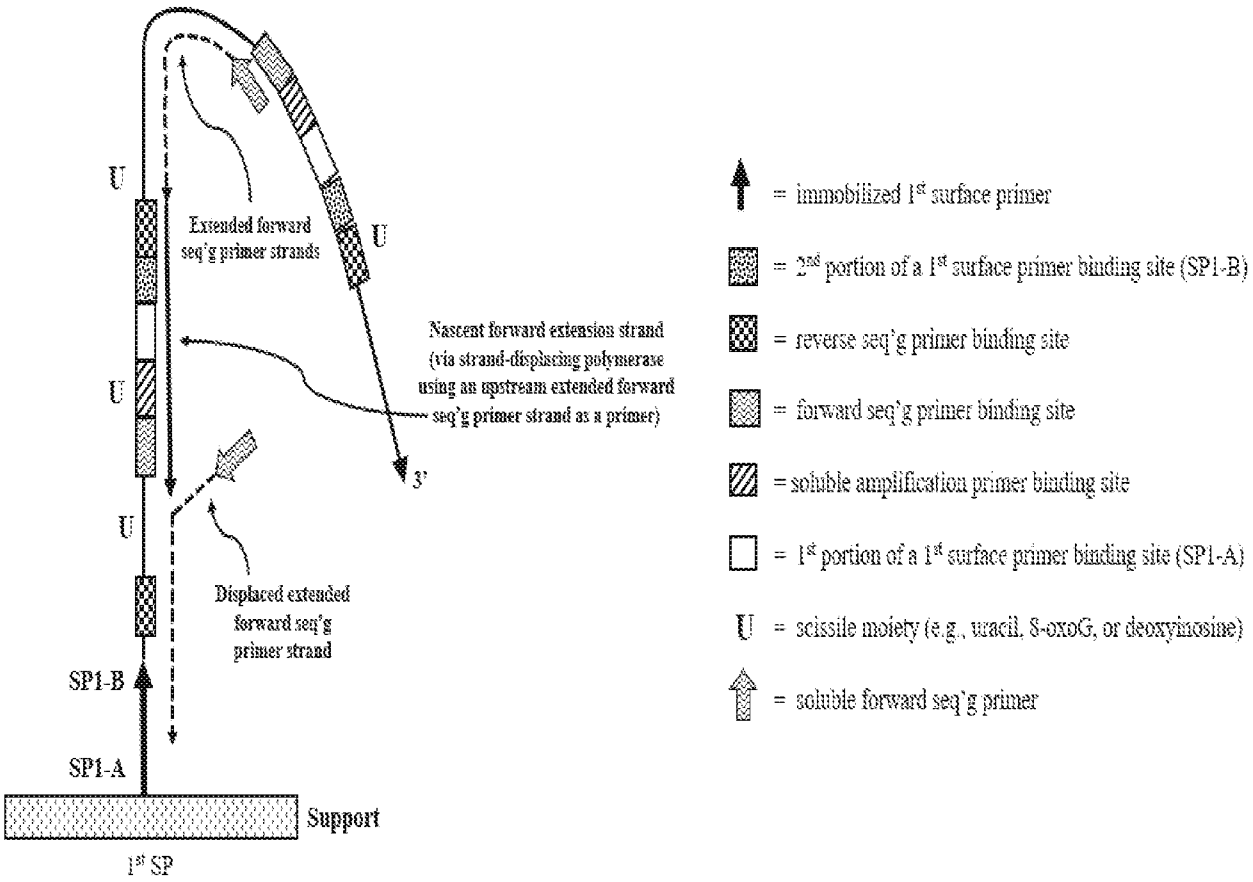

U
U
U
U
Extended forward seq'g primer strands
Nascent forward extension strand (via strand-displacing polymerase using an upstream extended forward seq'g primer strand as a primer)
3'
Displaced extended forward seq'g primer strand
SP1-B
SP1-A
Support
1st SP
= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble forward seq'g primer

FIG. 63

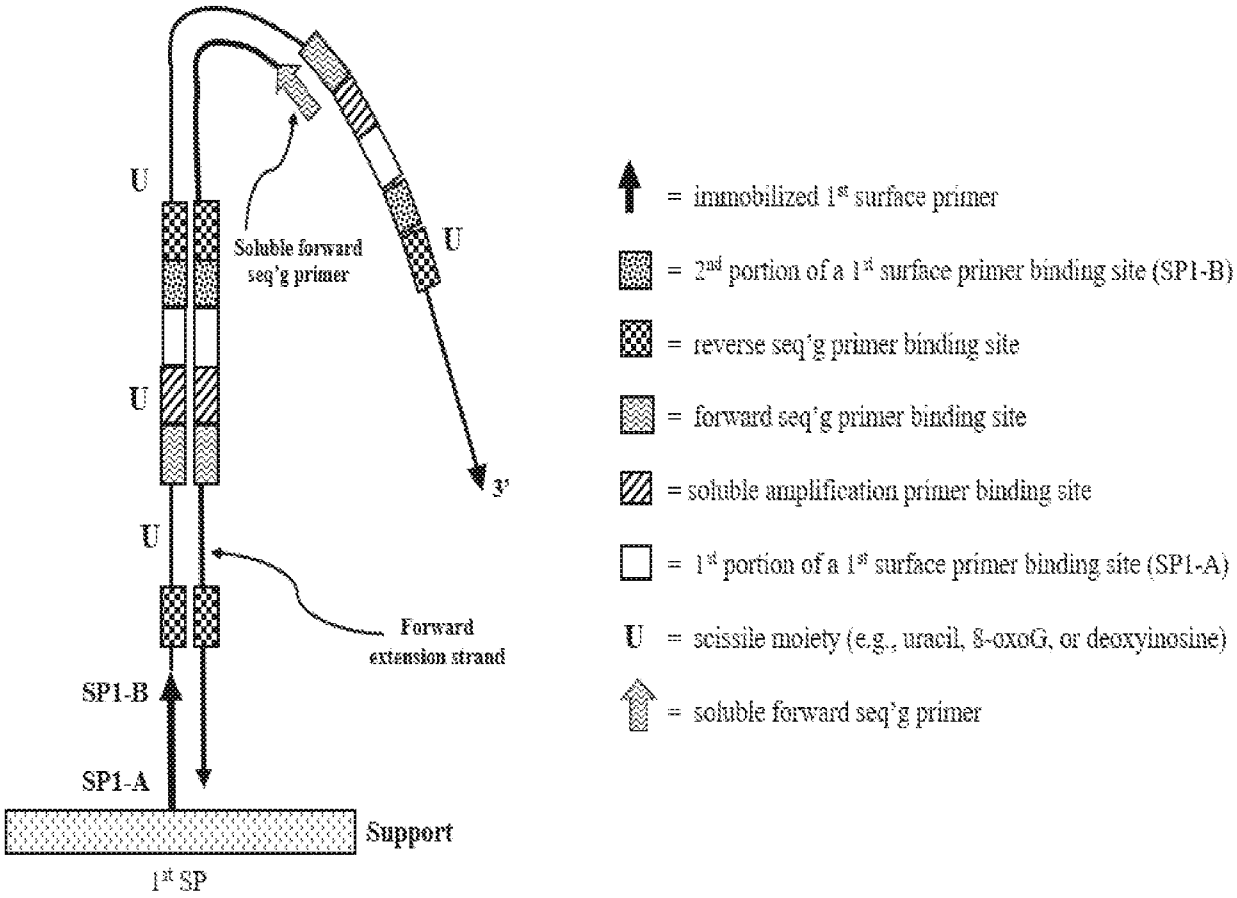

U
U
U
U
Soluble forward seq'g primer
3'
Forward extension strand
SP1-B
SP1-A
Support
1st SP
= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble forward seq'g primer

FIG. 64

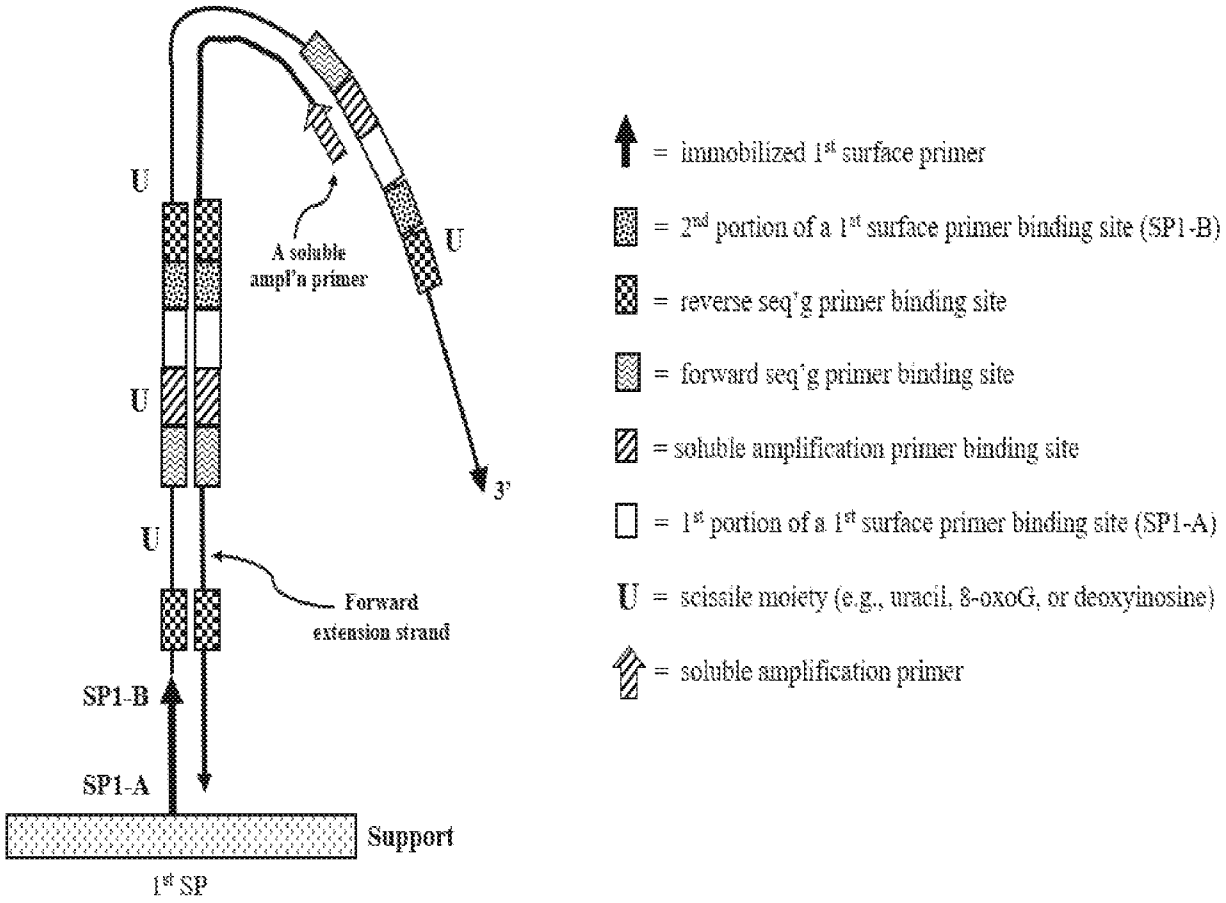

U
U
U
U
A soluble ampl'n primer
3'
Forward extension strand
SP1-B
SP1-A
Support
1st SP
= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble amplification primer

FIG. 65

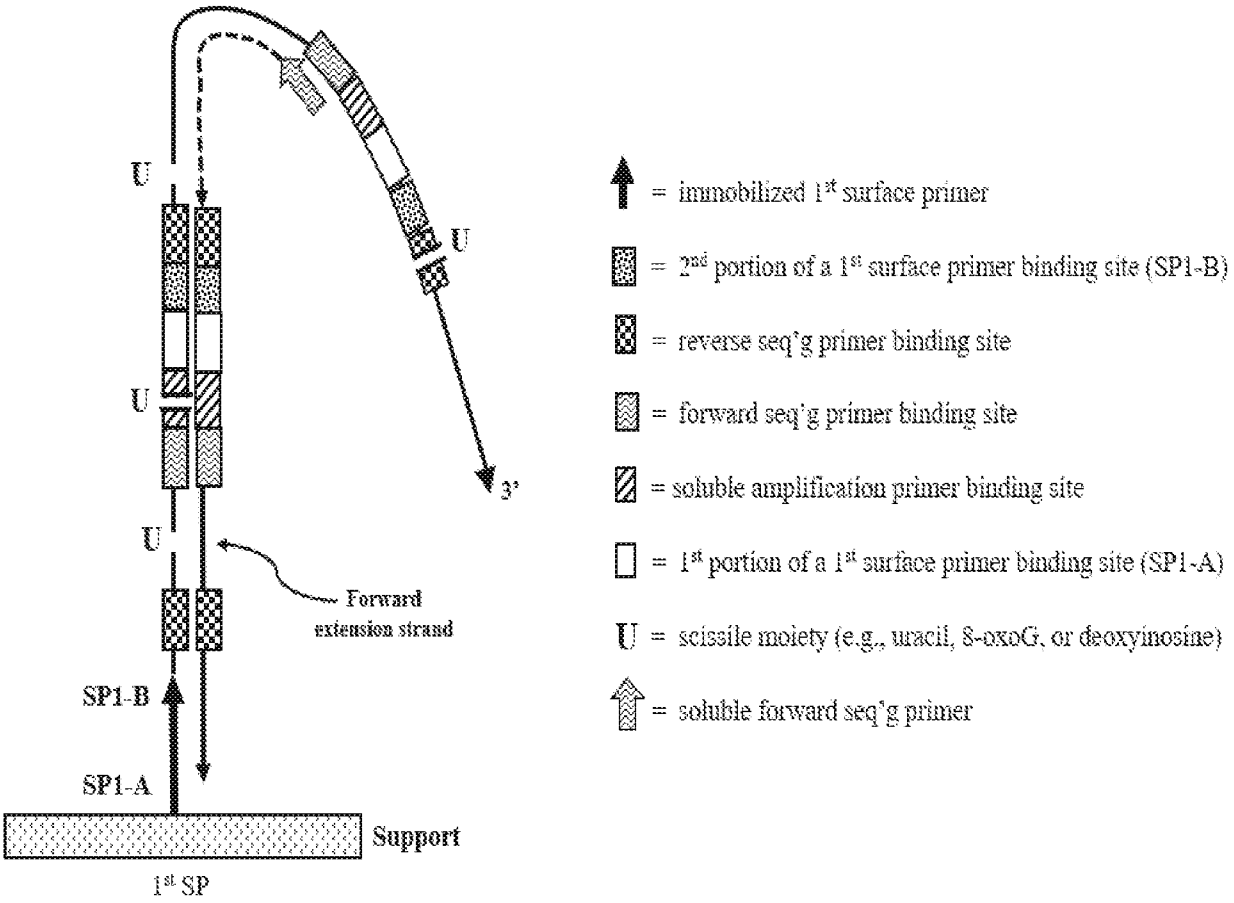

U
U
U
U
3'
Forward extension strand
SP1-B
SP1-A
Support
1st SP
= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble forward seq'g primer

FIG. 66

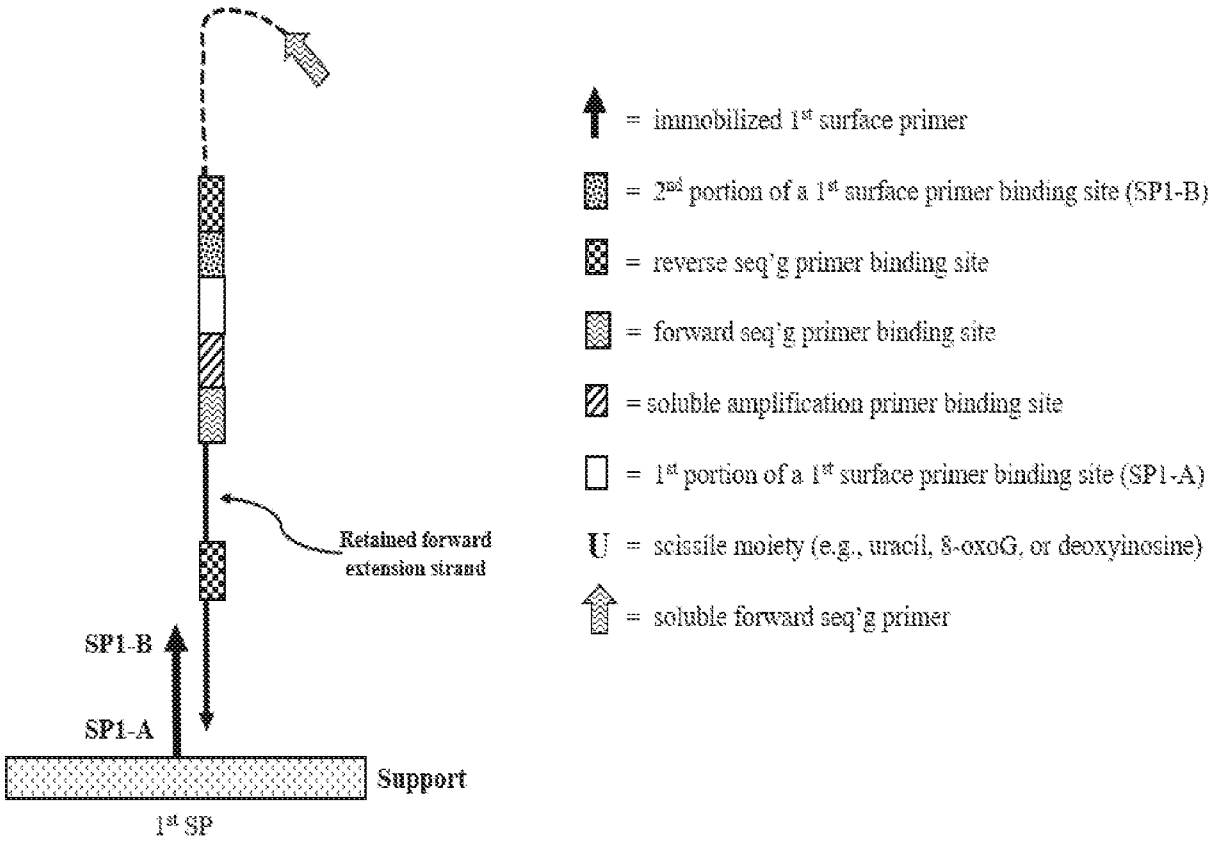

Retained forward extension strand
SP1-B
SP1-A
Support
1st SP
= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble forward seq'g primer

FIG. 67

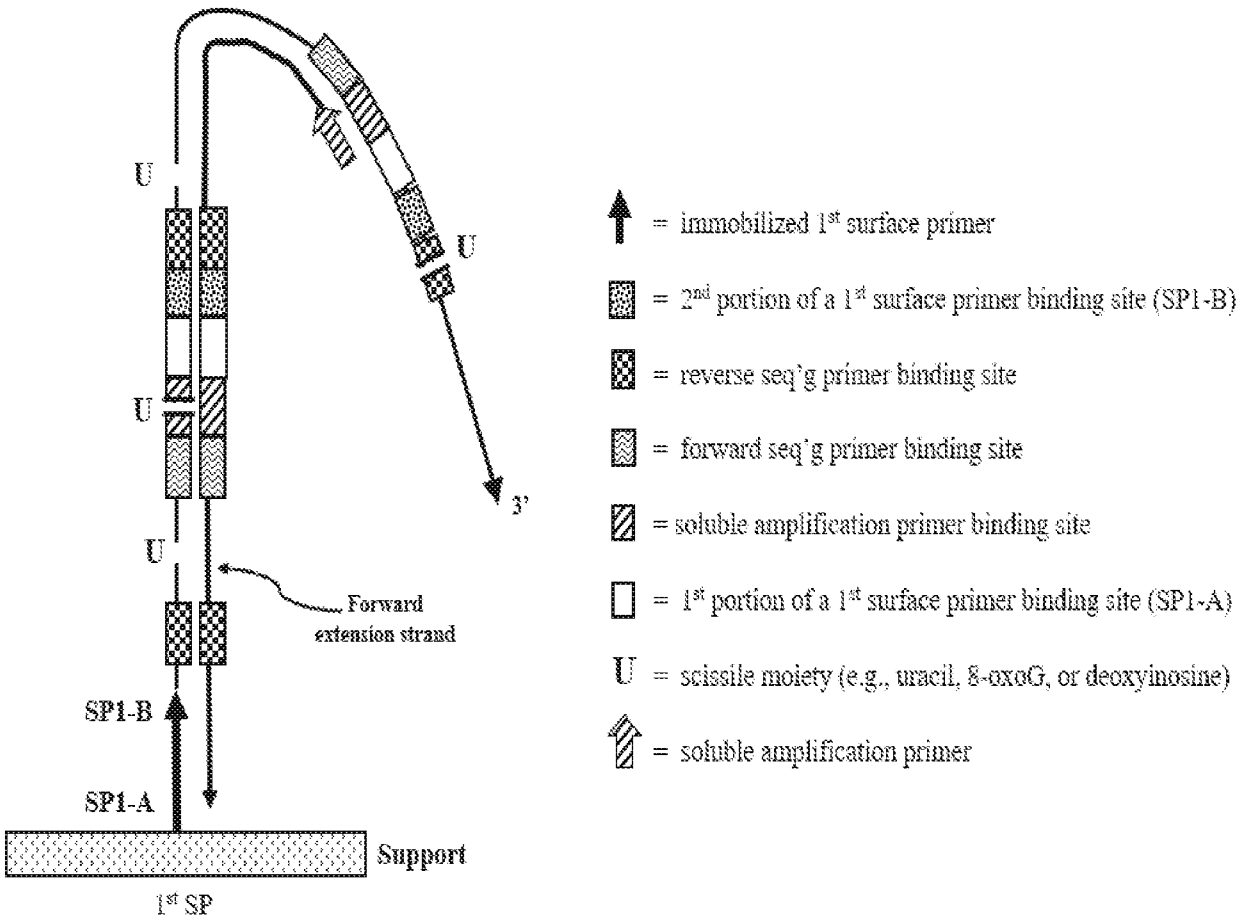

U
U
U
U
3'
Forward extension strand
SP1-B
SP1-A
Support
1st SP
= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble amplification primer

FIG. 68

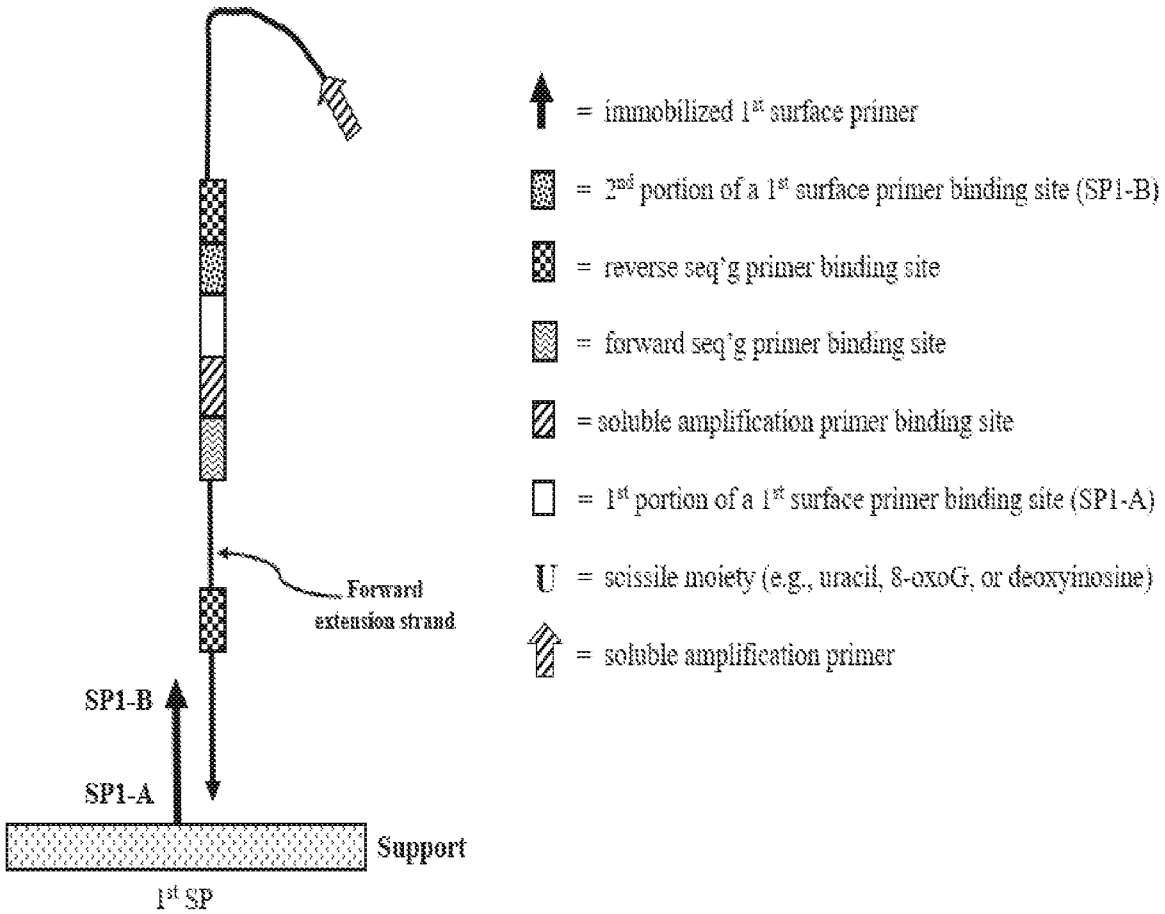

= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= soluble amplification primer
Forward extension strand
SP1-B
SP1-A
Support
1st SP

FIG. 69

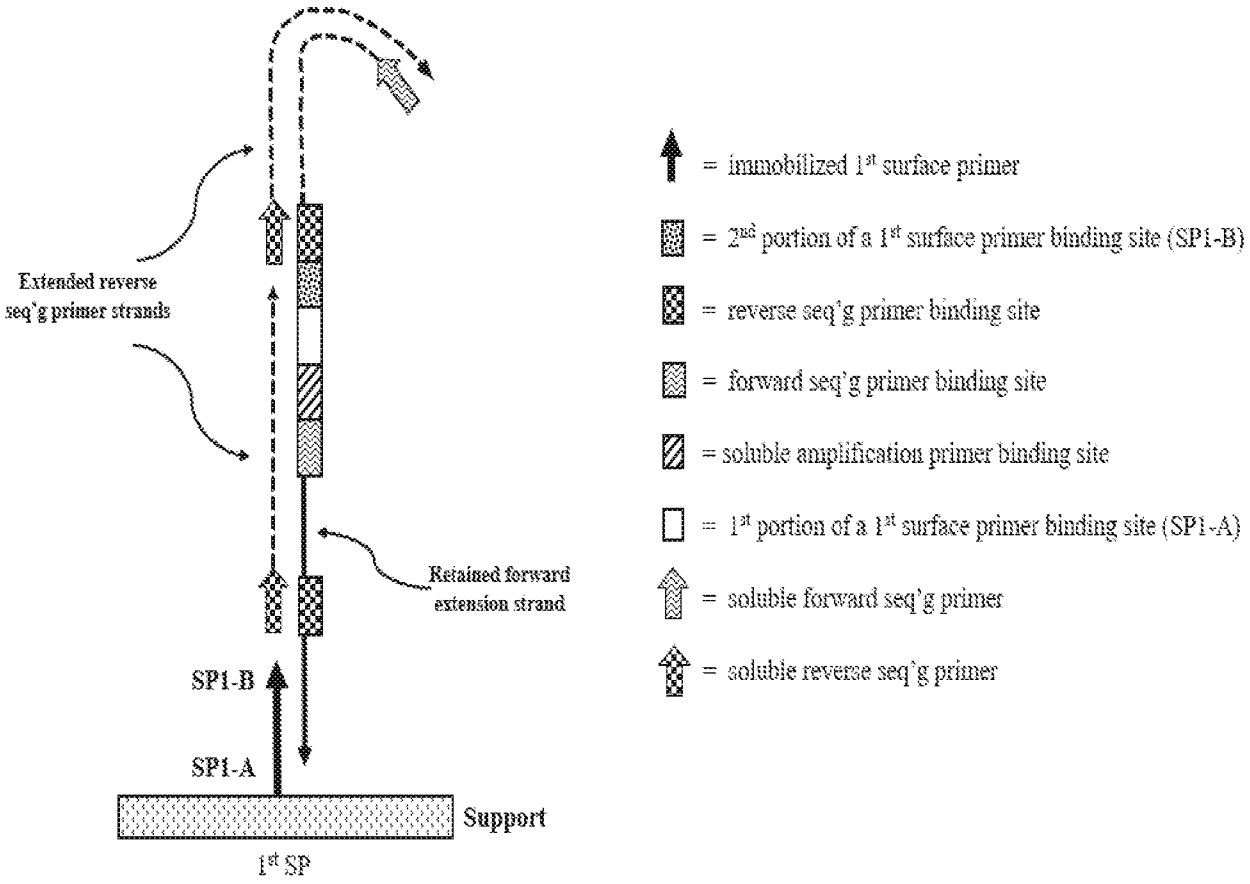

Extended reverse seq'g primer strands
Retained forward extension strand
SP1-B
SP1-A
Support
1st SP
= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)
= soluble forward seq'g primer
= soluble reverse seq'g primer

FIG. 70

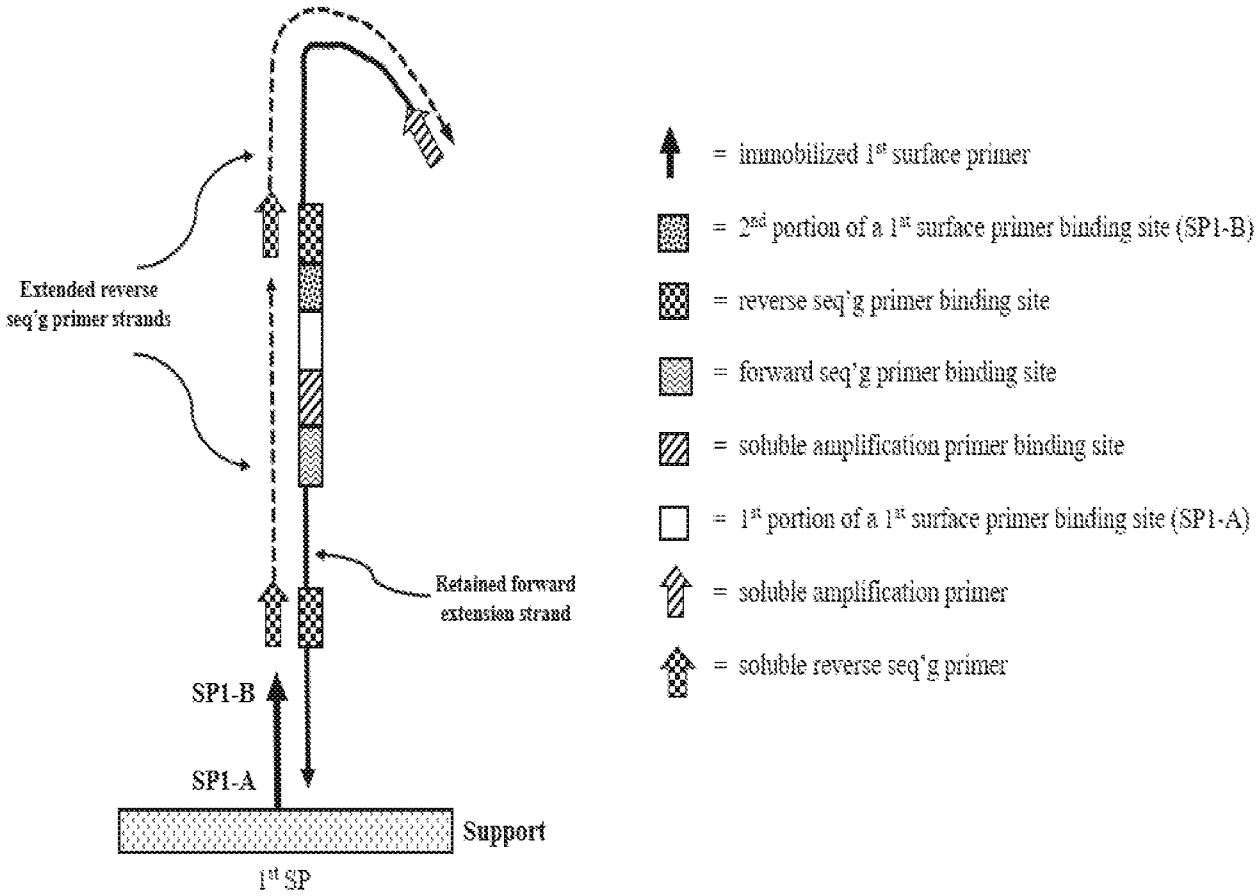

Extended reverse seq'g primer strands
Retained forward extension strand
SP1-B
SP1-A
Support
1st SP
= immobilized 1st surface primer
= 2nd portion of a 1st surface primer binding site (SP1-B)
= reverse seq'g primer binding site
= forward seq'g primer binding site
= soluble amplification primer binding site
= 1st portion of a 1st surface primer binding site (SP1-A)
= soluble amplification primer
= soluble reverse seq'g primer

FIG. 71

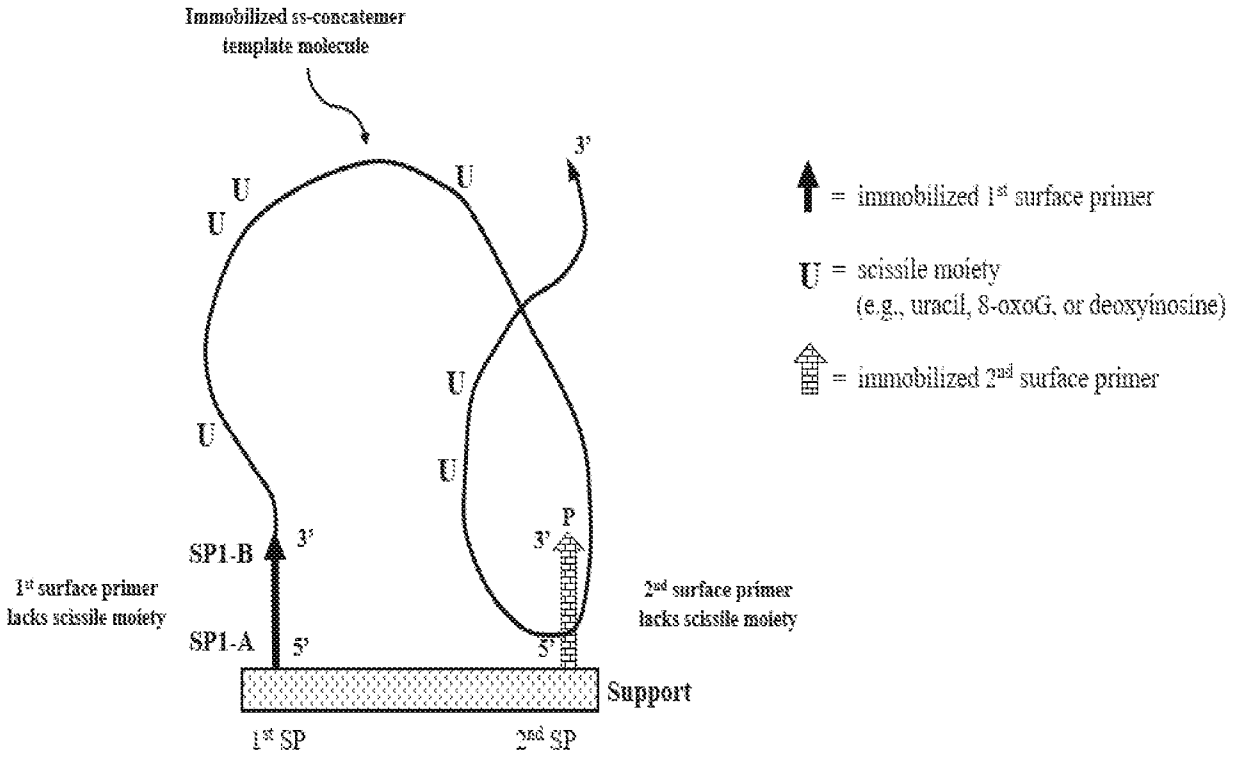

Immobilized ss-concatemer template molecule
3'
U
U
U
U
U
U
U
P
3'
3'
SP1-B
SP1-A
5'
5'
1st surface primer lacks scissile moiety
2nd surface primer lacks scissile moiety
Support
1st SP
2nd SP
= immobilized 1st surface primer
U = scissile moiety (e.g., uracil, 8-oxoG, or deoxyinosine)
= immobilized 2nd surface primer

FIG. 72

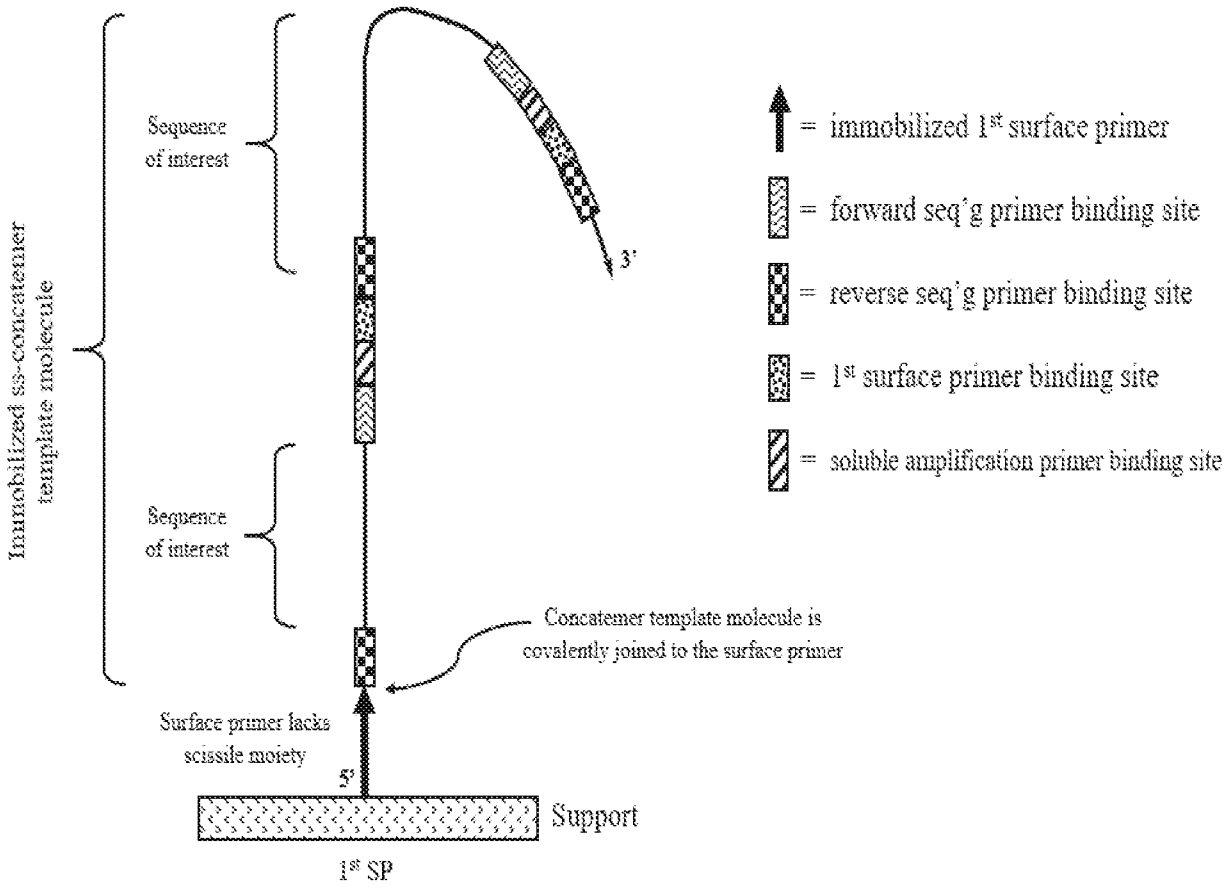

Immobilized ss-concatemer template molecule
Sequence of interest
Sequence of interest
3'
Concatemer template molecule is covalently joined to the surface primer
Surface primer lacks scissile moiety
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site

FIG. 73

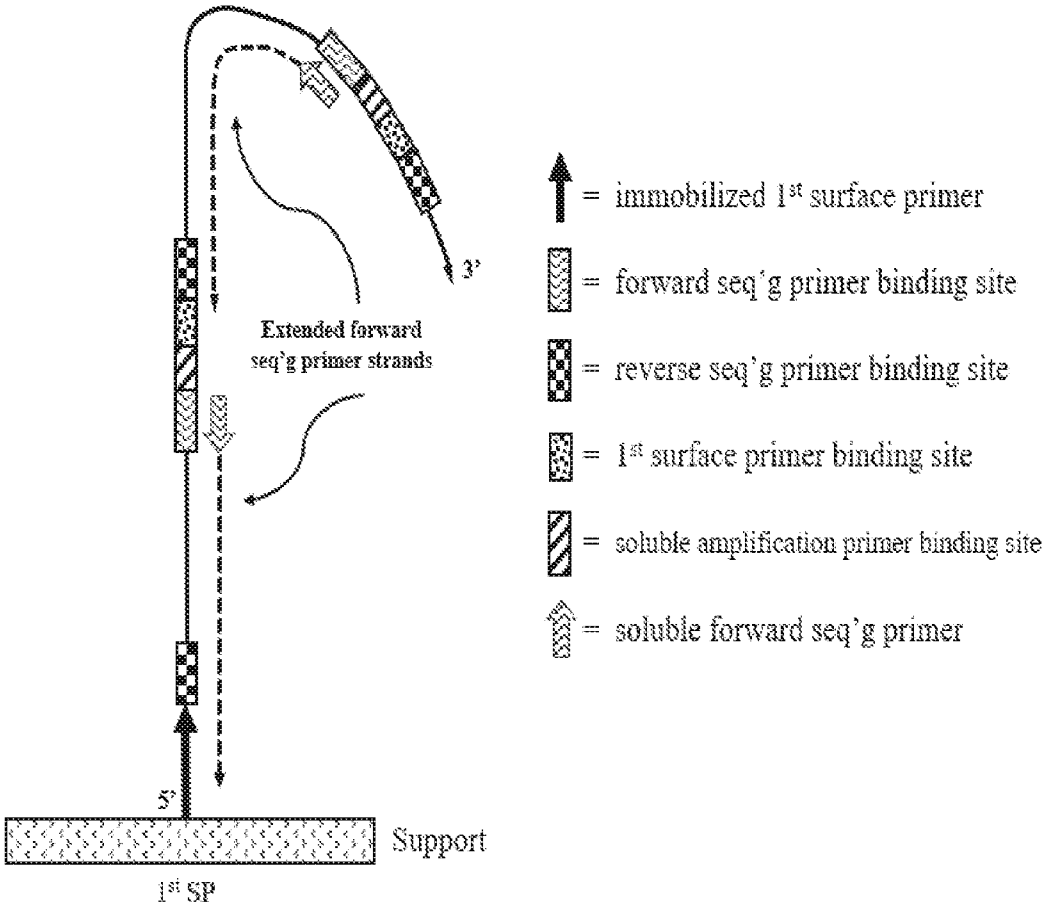

3'
Extended forward seq'g primer strands
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
= soluble forward seq'g primer

FIG. 74

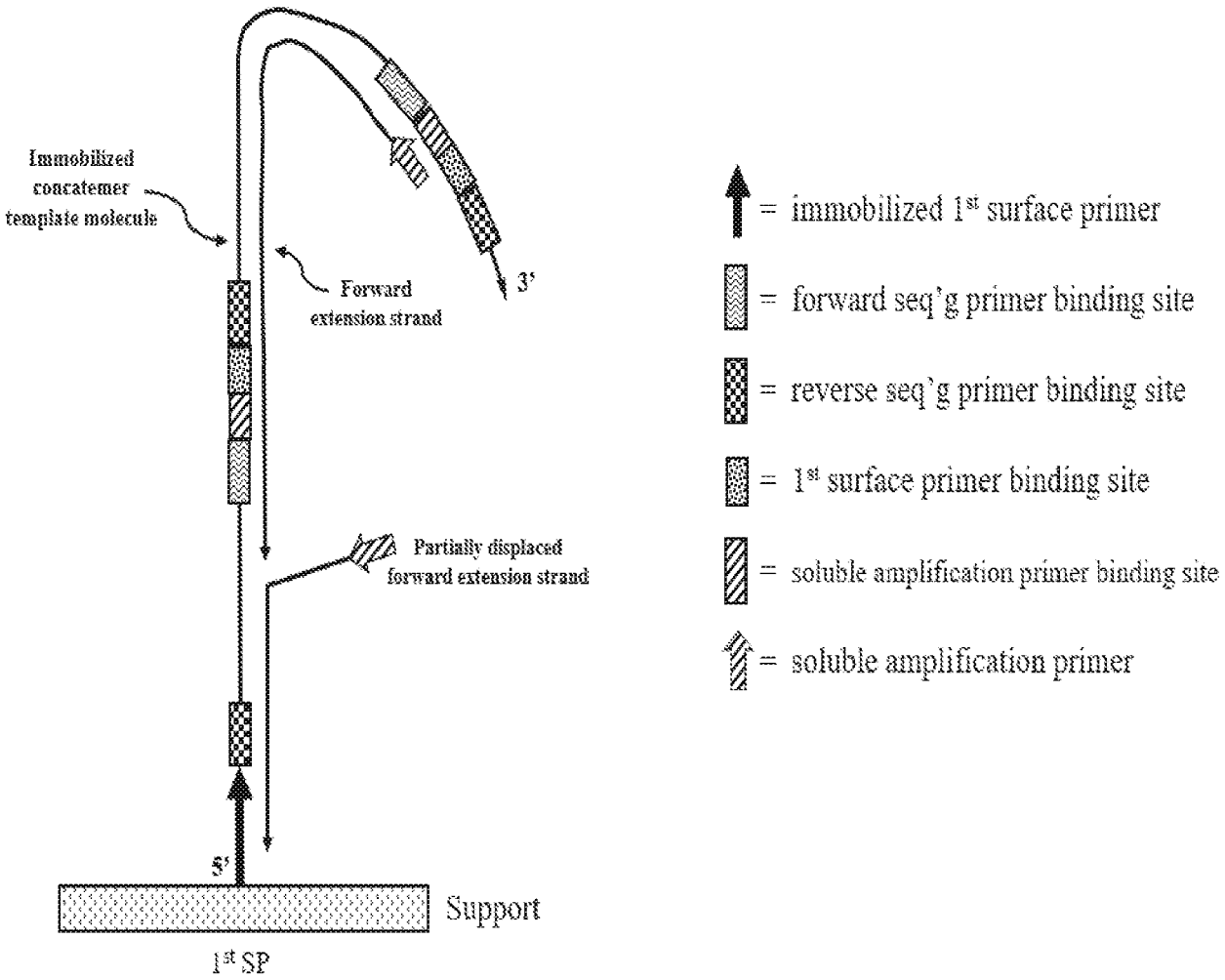

Immobilized concatemer template molecule
Forward extension strand
3'
Partially displaced forward extension strand
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
= soluble amplification primer

FIG. 75

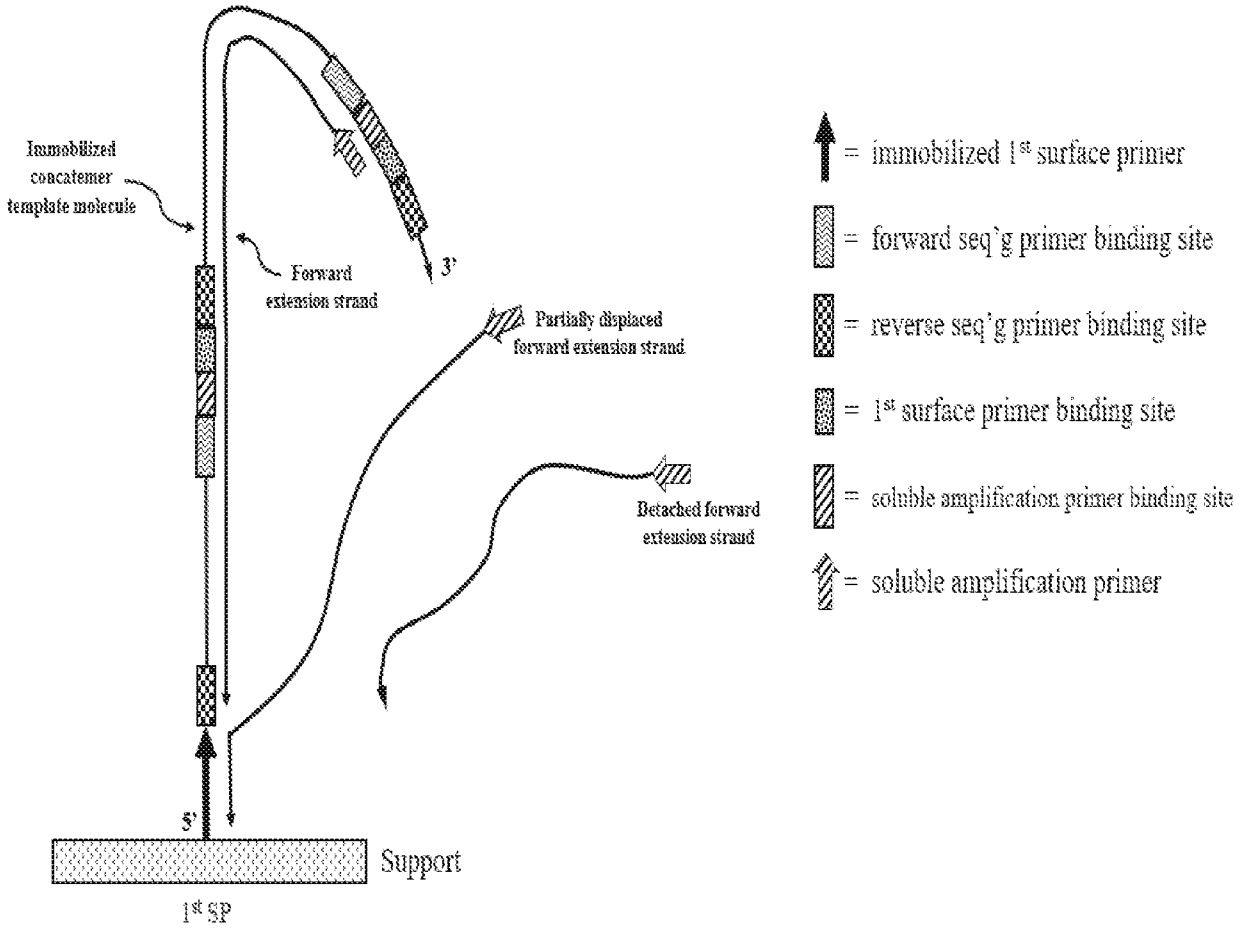

Immobilized concatemer template molecule
Forward extension strand
3'
Partially displaced forward extension strand
Detached forward extension strand
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
= soluble amplification primer

FIG. 76

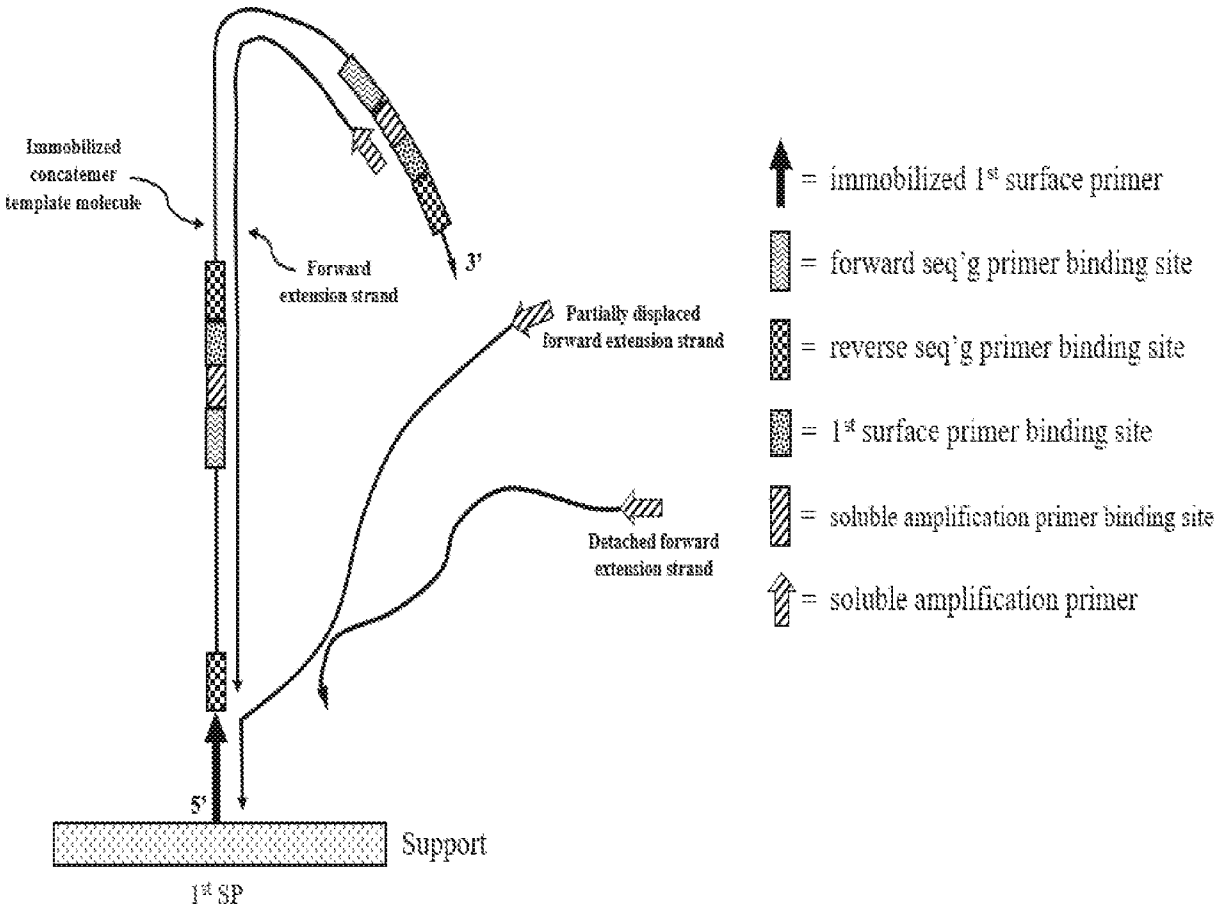

Immobilized concatemer template molecule
Forward extension strand
3'
Partially displaced forward extension strand
Detached forward extension strand
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
= soluble amplification primer

FIG. 77

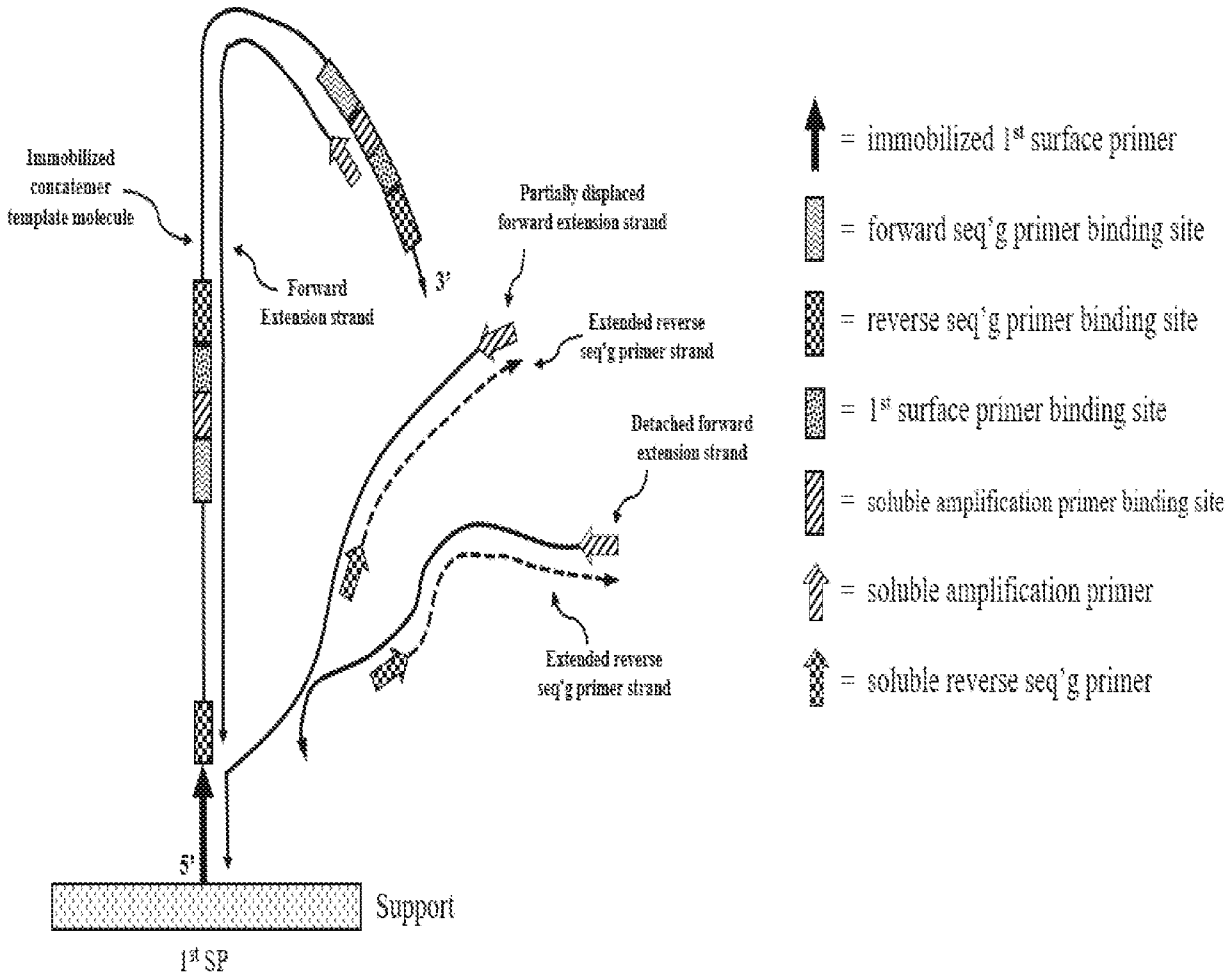

Immobilized
concatemer
template molecule
Forward
Extension strand
3'
Partially displaced
forward extension strand
Extended reverse
seq'g primer strand
Detached forward
extension strand
Extended reverse
seq'g primer strand
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
= soluble amplification primer
= soluble reverse seq'g primer

FIG. 78

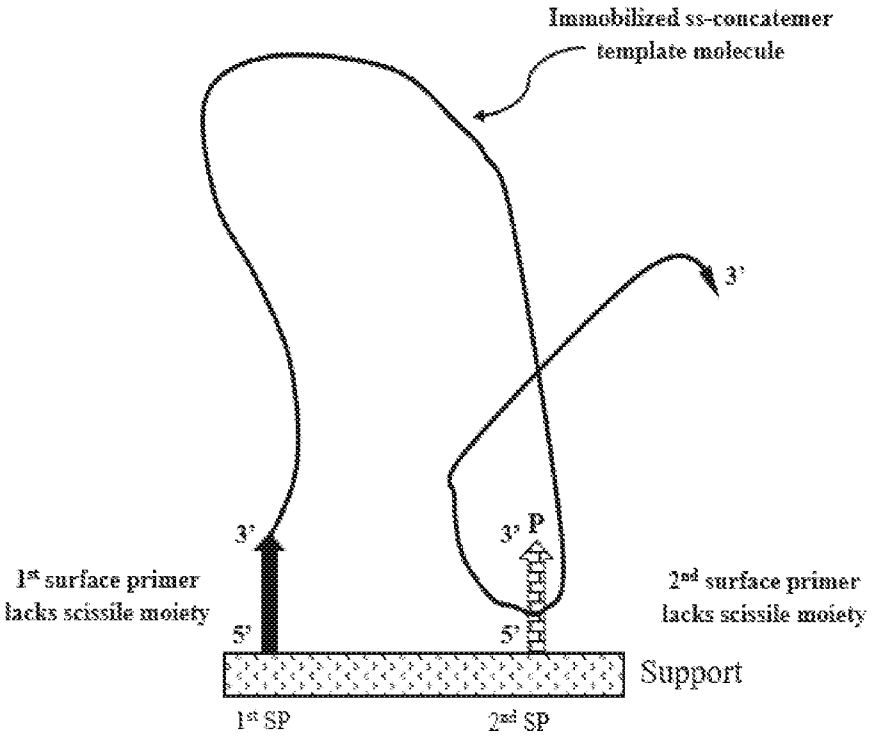

Immobilized ss-concatemer
template molecule
3'
3'
P
3'
1st surface primer
lacks scissile moiety
2nd surface primer
lacks scissile moiety
5'
5'
Support
1st SP
2nd SP

FIG. 79

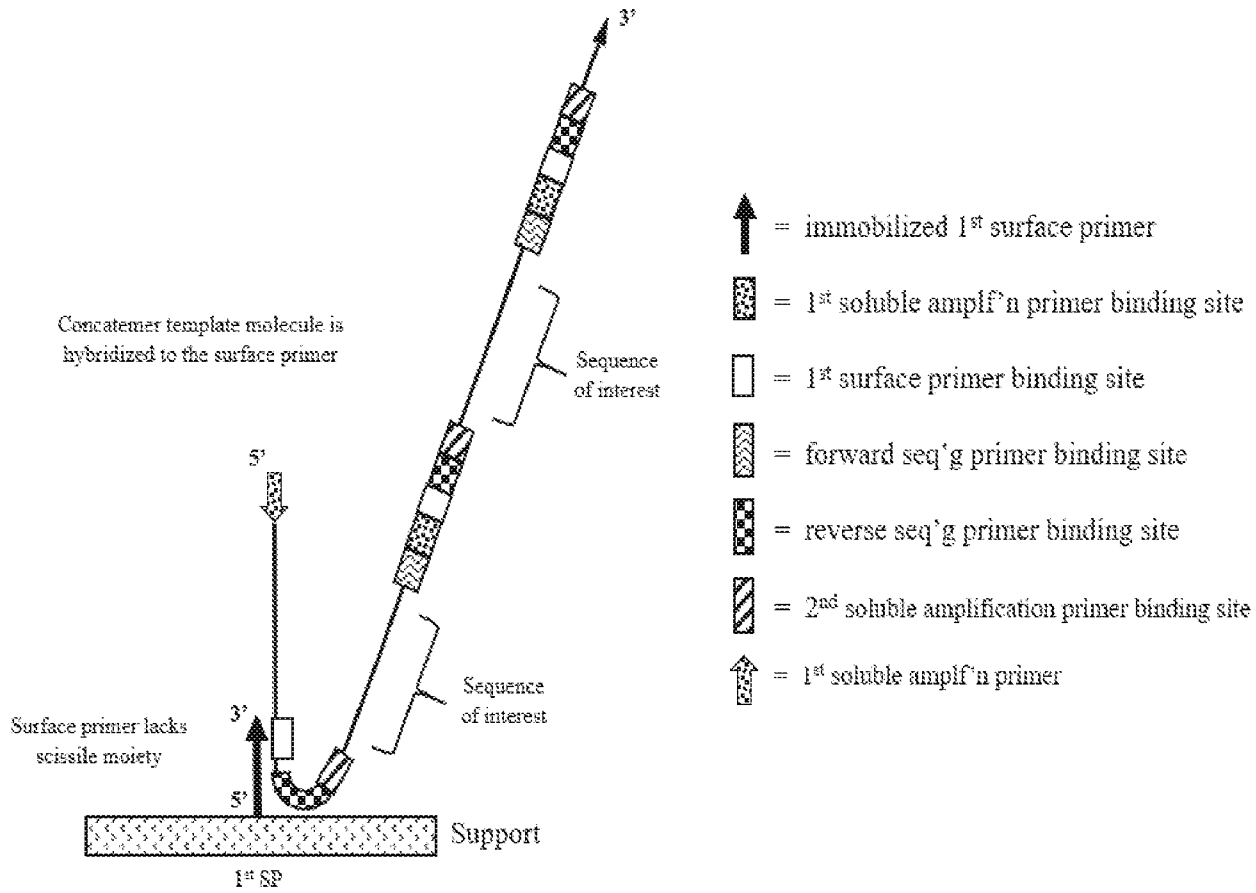

3'
Concatemer template molecule is hybridized to the surface primer
Sequence of interest
5'
Sequence of interest
Surface primer lacks scissile moiety
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
= 1st soluble amplf'n primer

FIG. 80

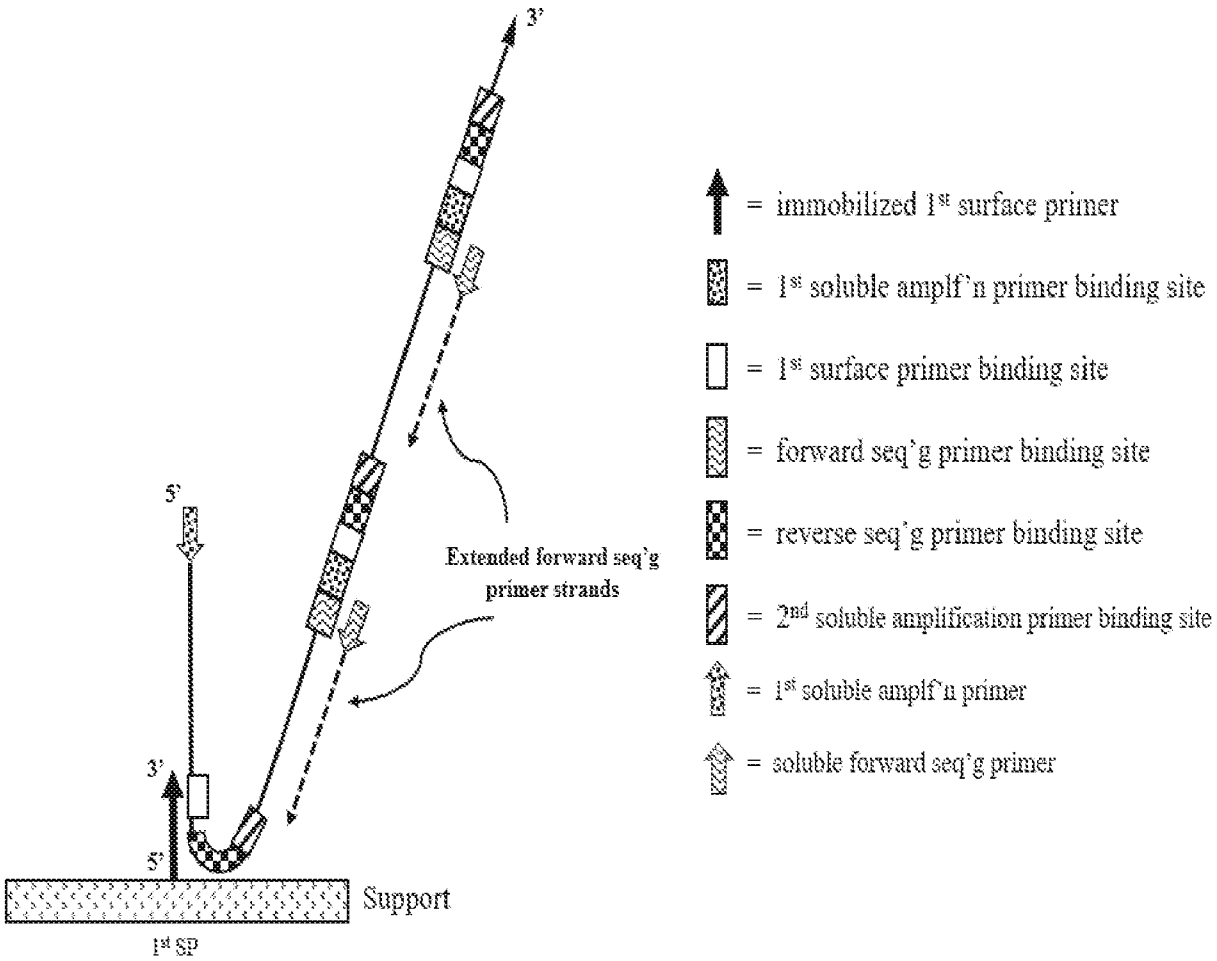

3'
5'
3'
5'
Extended forward seq'g primer strands
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
= 1st soluble amplf'n primer
= soluble forward seq'g primer

FIG. 81

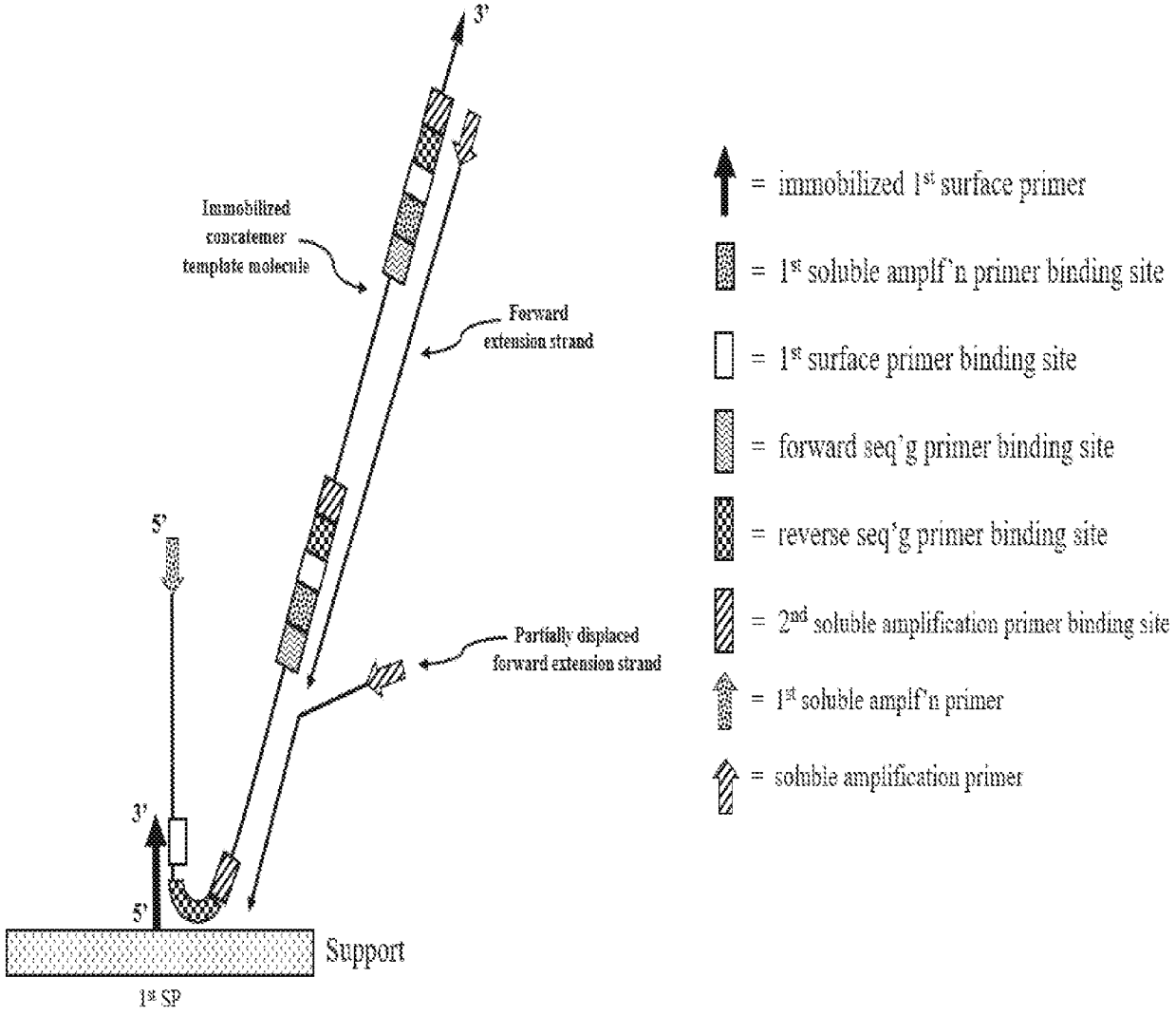

3'
Immobilized concatemer template molecule
Forward extension strand
5'
Partially displaced forward extension strand
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
= 1st soluble amplf'n primer
= soluble amplification primer

FIG. 82

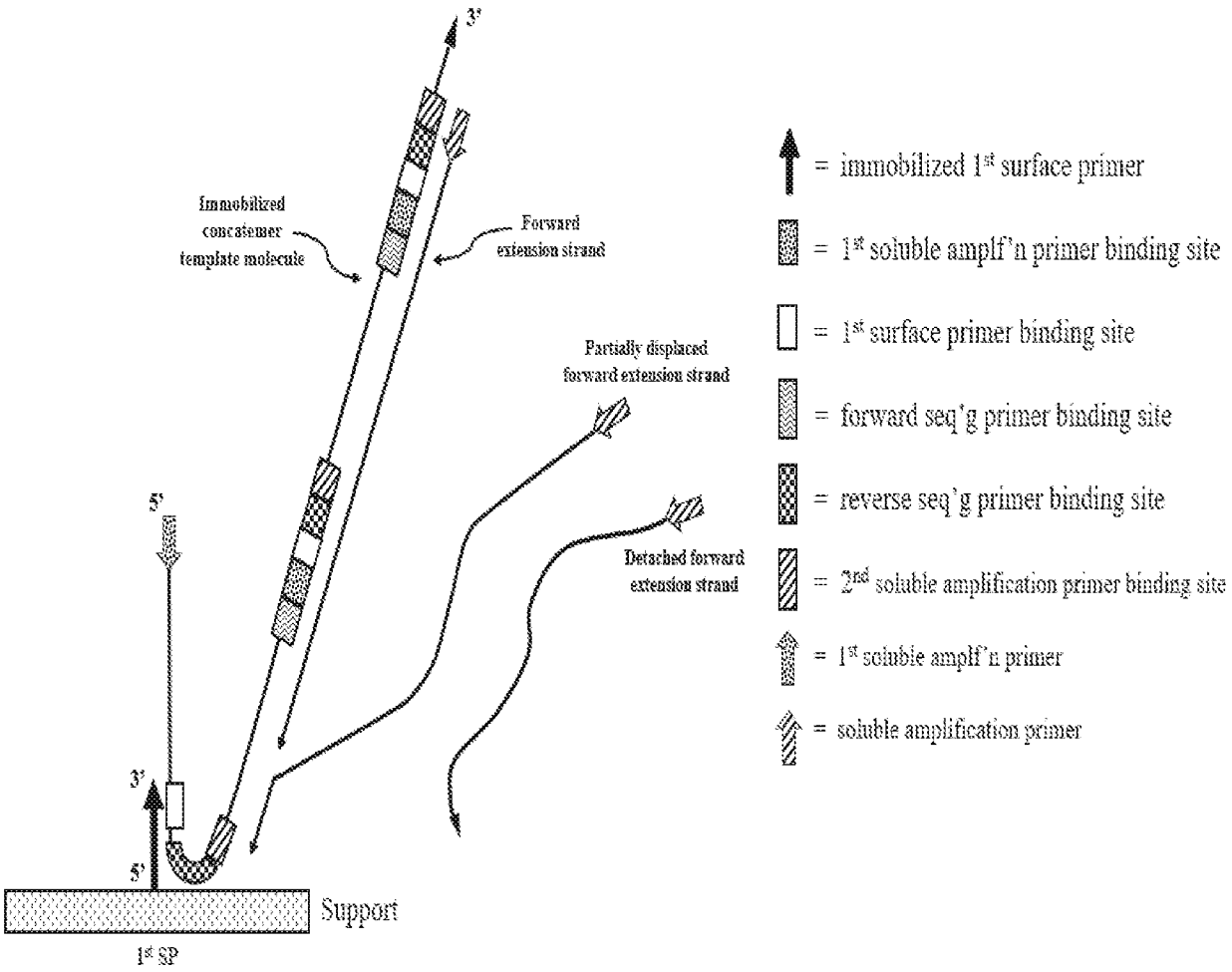

3'
Immobilized concatemer template molecule
Forward extension strand
Partially displaced forward extension strand
Detached forward extension strand
5'
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
= 1st soluble amplf'n primer
= soluble amplification primer

FIG. 83

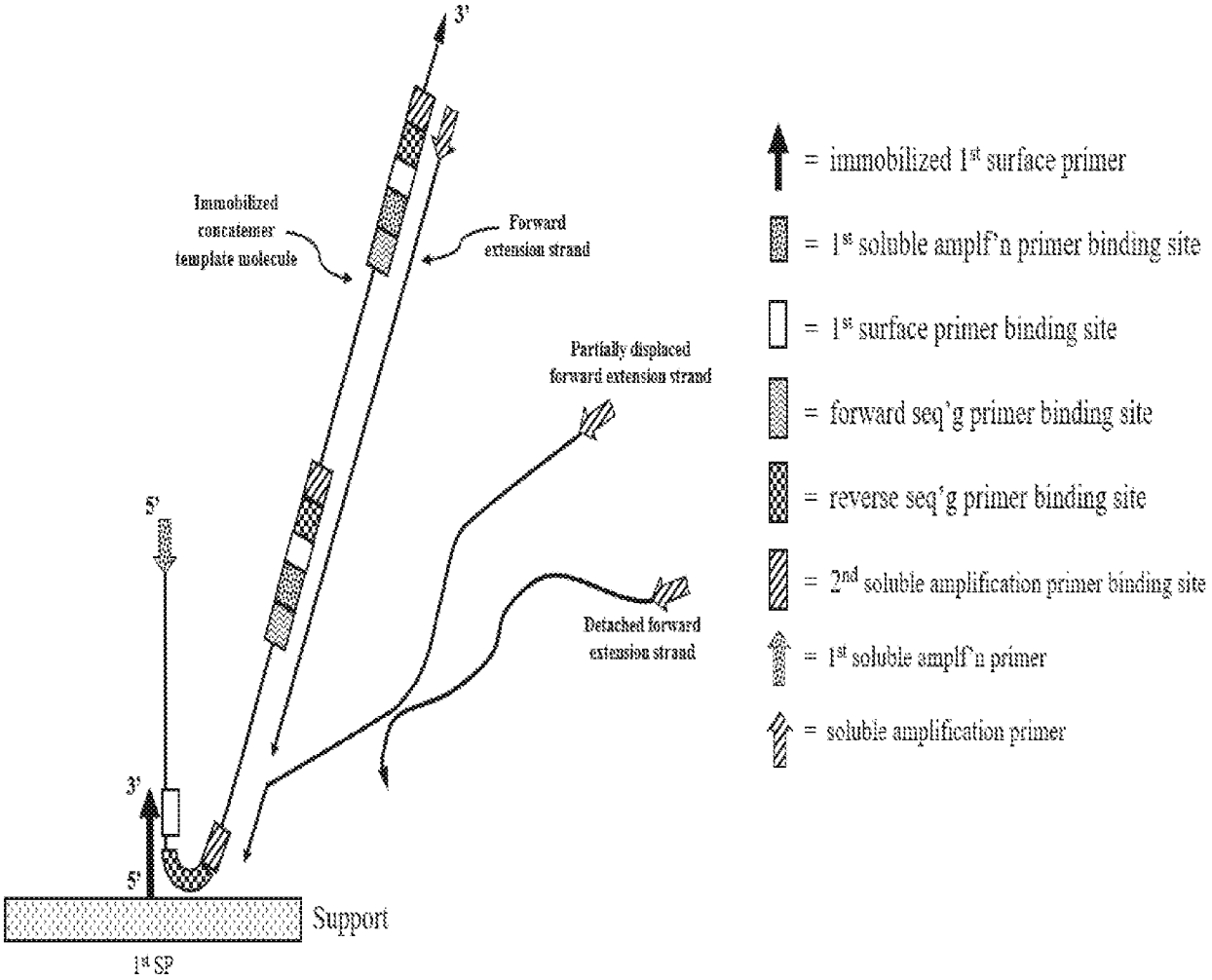

3'
Immobilized concatemer template molecule
Forward extension strand
Partially displaced forward extension strand
Detached forward extension strand
5'
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
= 1st soluble amplf'n primer
= soluble amplification primer

FIG. 84

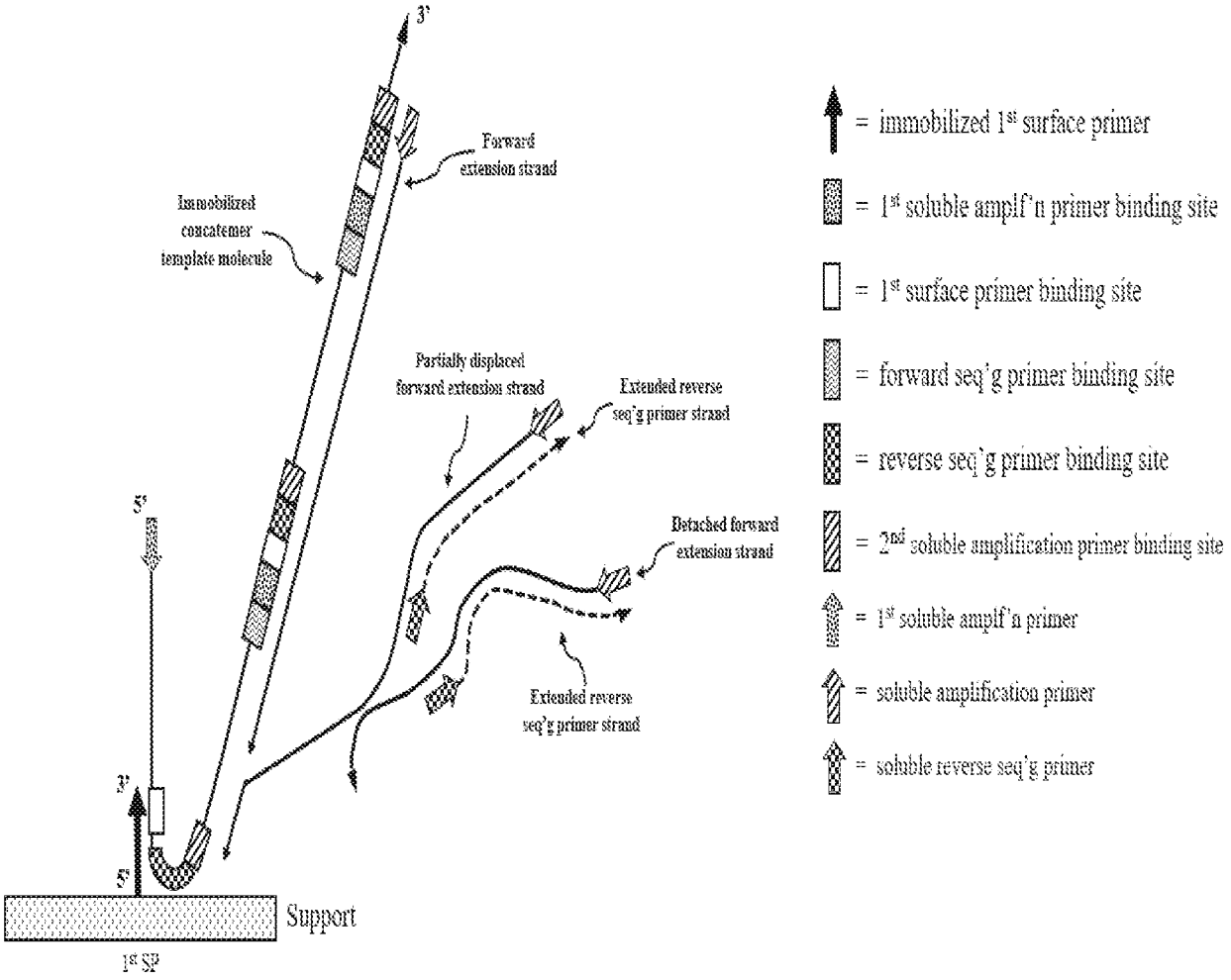

3'
Forward extension strand
Immobilized concatemer template molecule
Partially displaced forward extension strand
Extended reverse seq'g primer strand
5'
Detached forward extension strand
Extended reverse seq'g primer strand
3'
5'
Support
1st SP
= immobilized 1st surface primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site
= 1st soluble amplf'n primer
= soluble amplification primer
= soluble reverse seq'g primer

FIG. 85

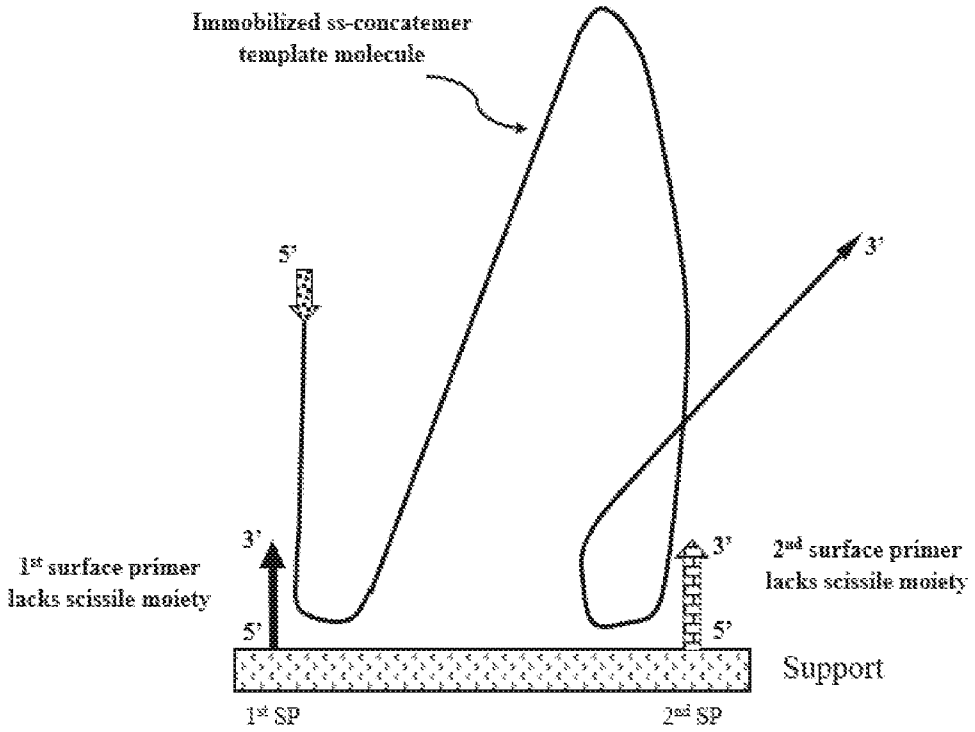

Immobilized ss-concatemer template molecule
5'
3'
3'
1st surface primer lacks scissile moiety
5'
3'
2nd surface primer lacks scissile moiety
5'
Support
1st SP
2nd SP

FIG. 86

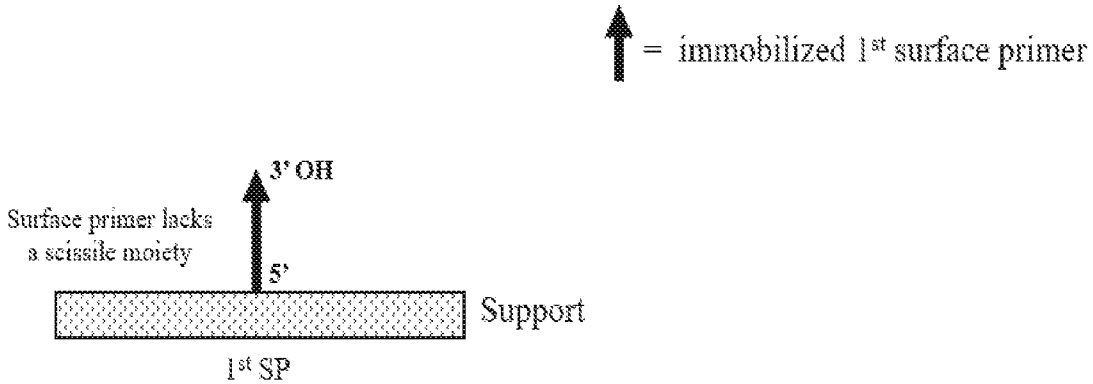

= immobilized 1st surface primer
3' OH
Surface primer lacks a scissile moiety
5'
Support
1st SP

FIG. 87

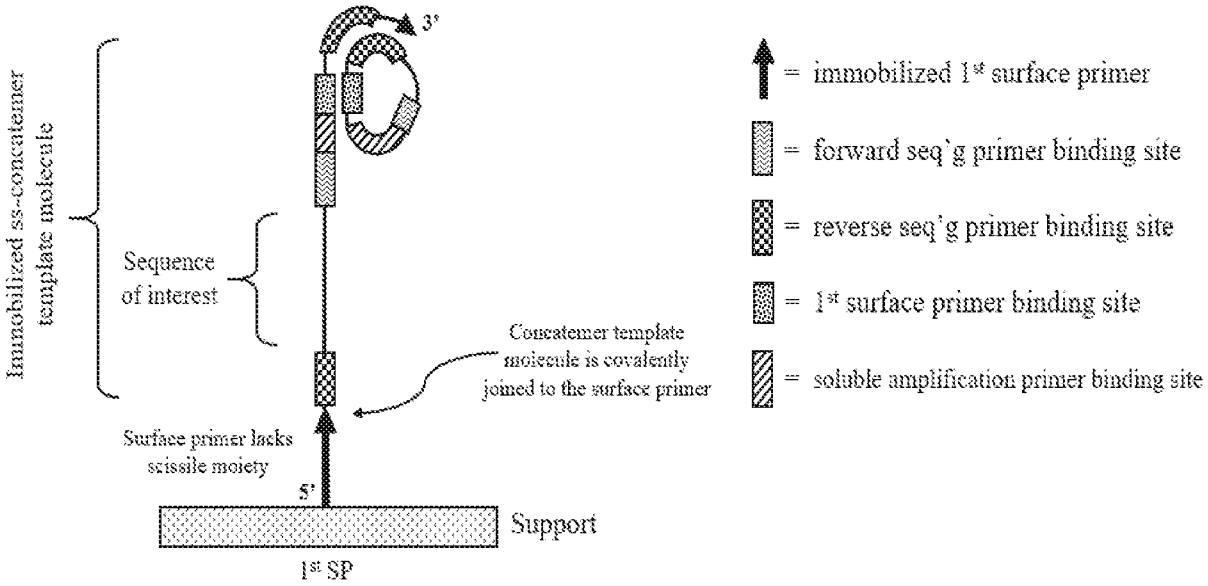

3'
Immobilized ss-concatemer template molecule
Sequence of interest
Concatemer template molecule is covalently joined to the surface primer
Surface primer lacks scissile moiety
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site

FIG. 88

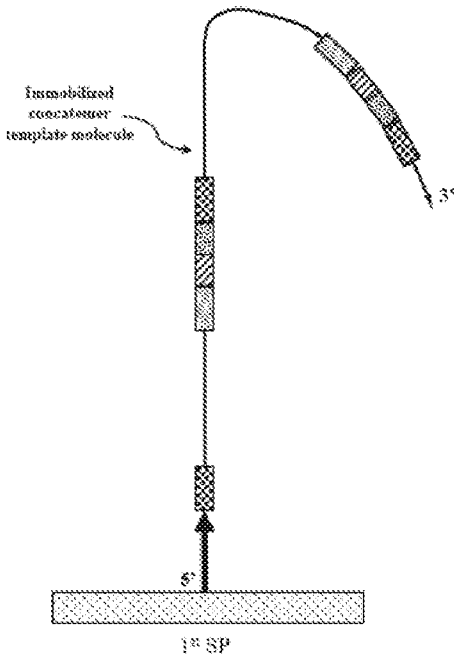


FIG. 89

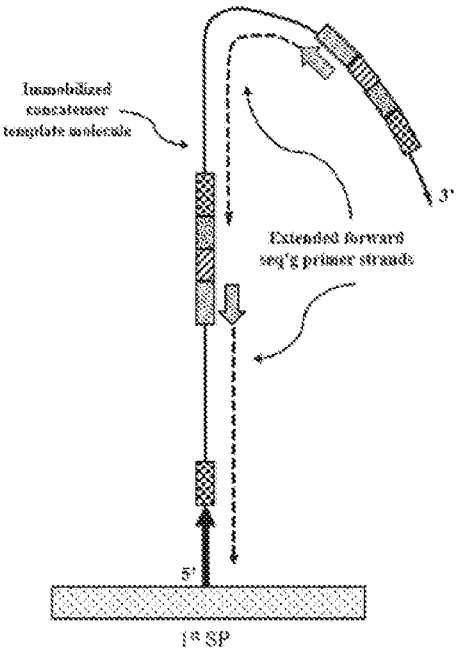


FIG. 90

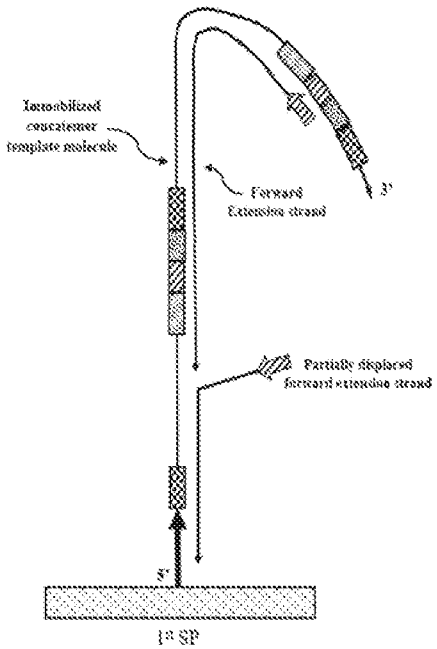


FIG. 91

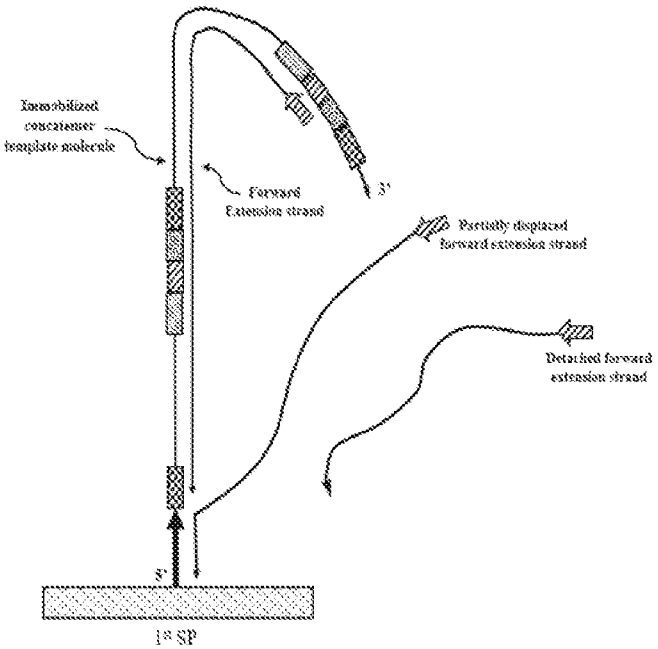


FIG. 92

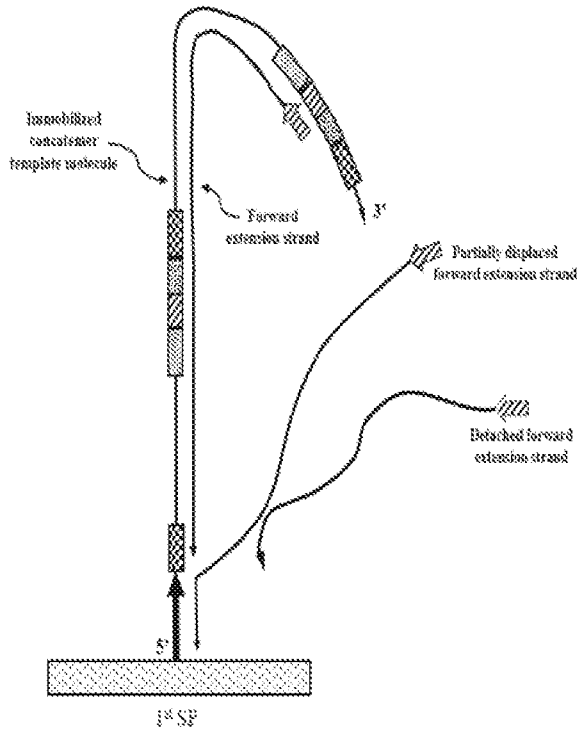

Immobilized concatemer template molecule
Forward extension strand
3'
Partially displaced forward extension strand
Detached forward extension strand
5'
dNTP

FIG. 93

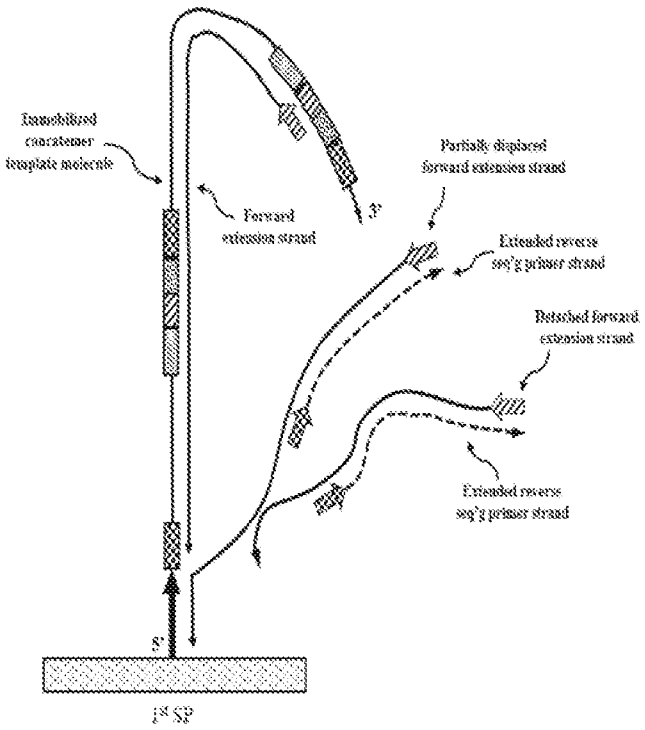

Immobilized concatemer template molecule
Forward extension strand
3'
Partially displaced forward extension strand
Extended reverse seq'g primer strand
Detached forward extension strand
Extended reverse seq'g primer strand
5'
dNTP

FIG. 94

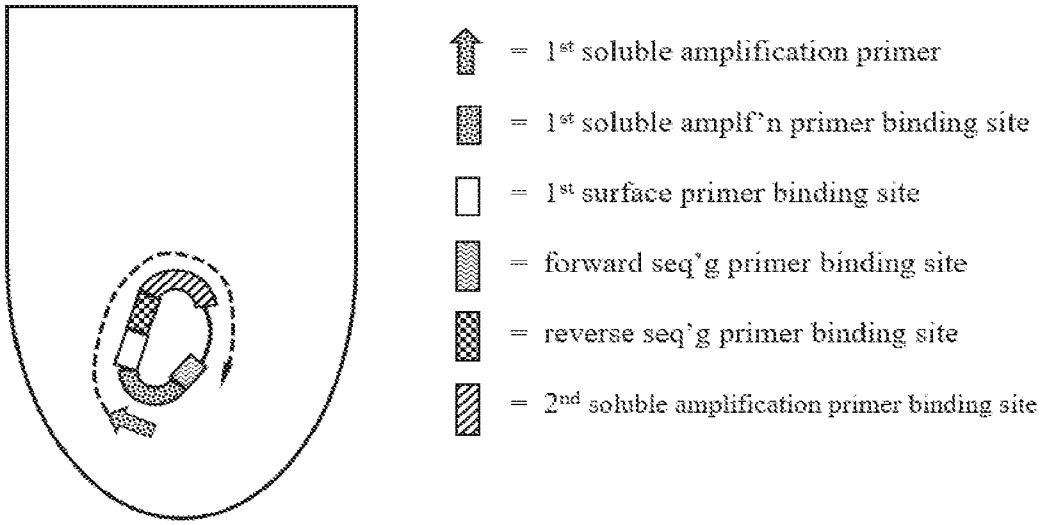

= 1st soluble amplification primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq`g primer binding site
= 2nd soluble amplification primer binding site

FIG. 95

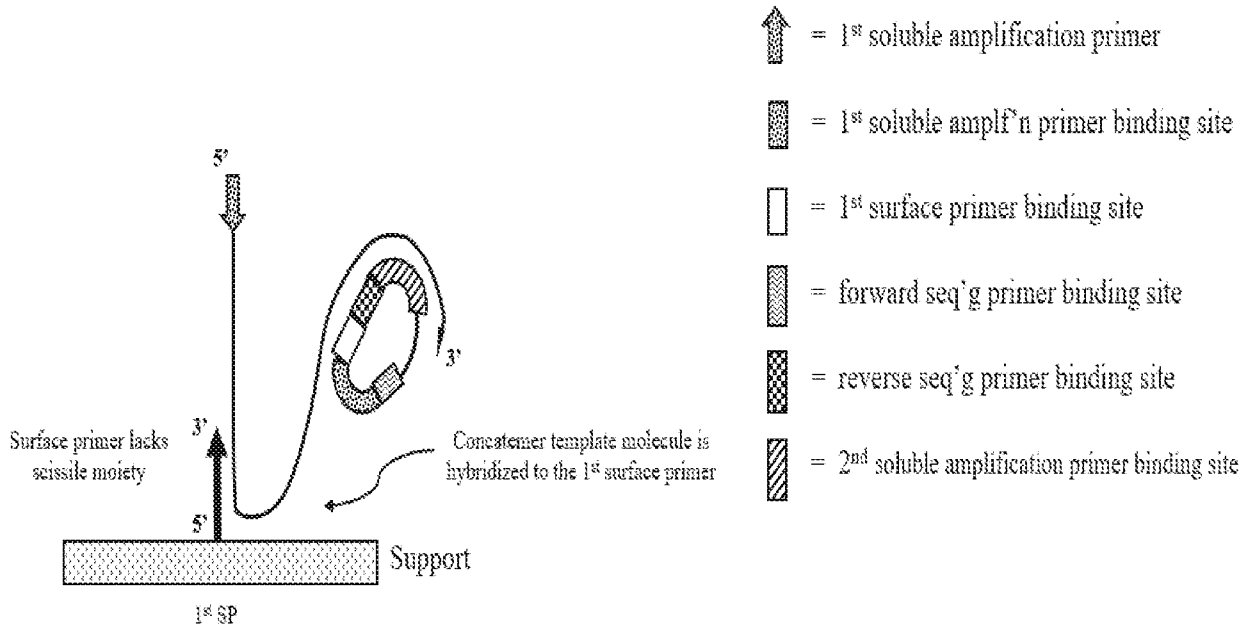

5'
3'
3'
5'
Surface primer lacks scissile moiety
Concatemer template molecule is hybridized to the 1st surface primer
Support
1st SP
= 1st soluble amplification primer
= 1st soluble amplf'n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site

FIG. 96

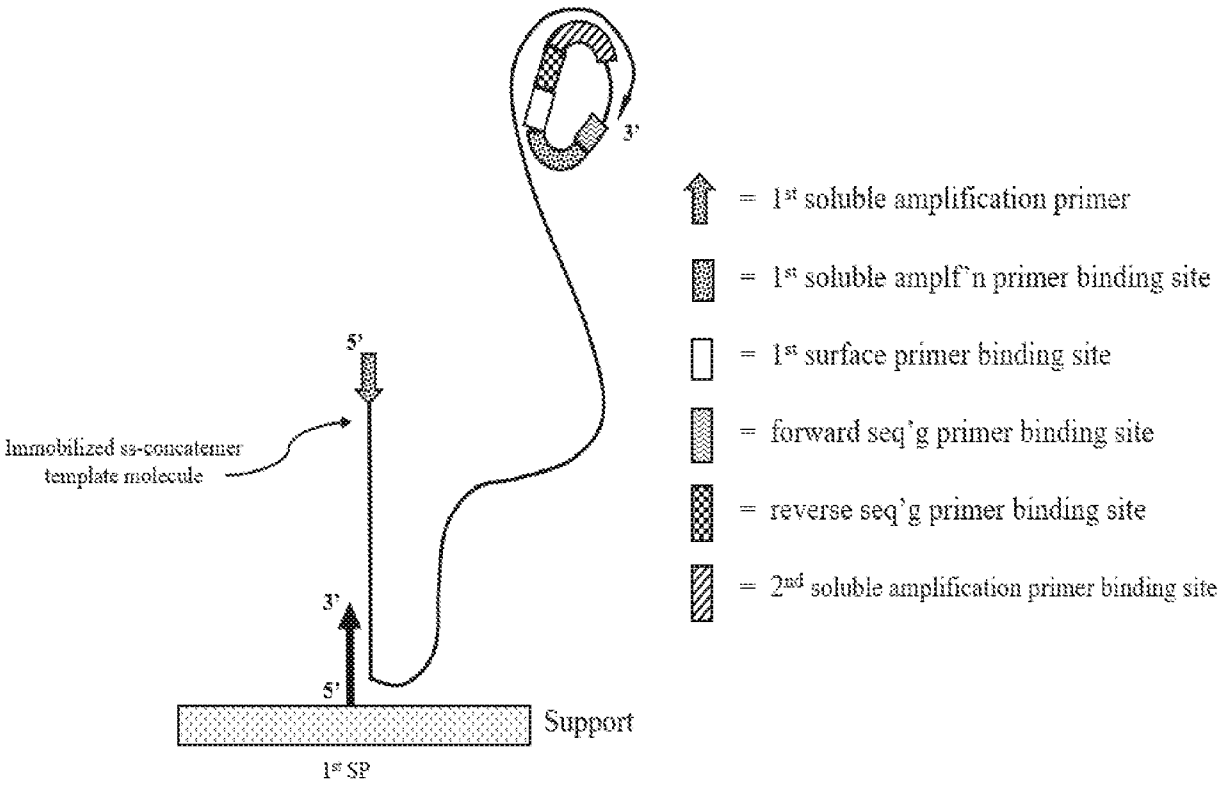

3'
5'
Immobilized ss-concatemer template molecule
3'
5'
Support
1st SP
= 1st soluble amplification primer
= 1st soluble amplf`n primer binding site
= 1st surface primer binding site
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 2nd soluble amplification primer binding site

FIG. 97

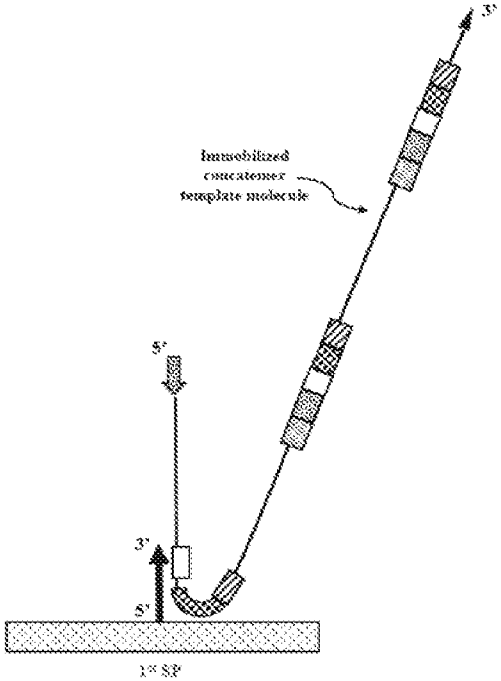


FIG. 98

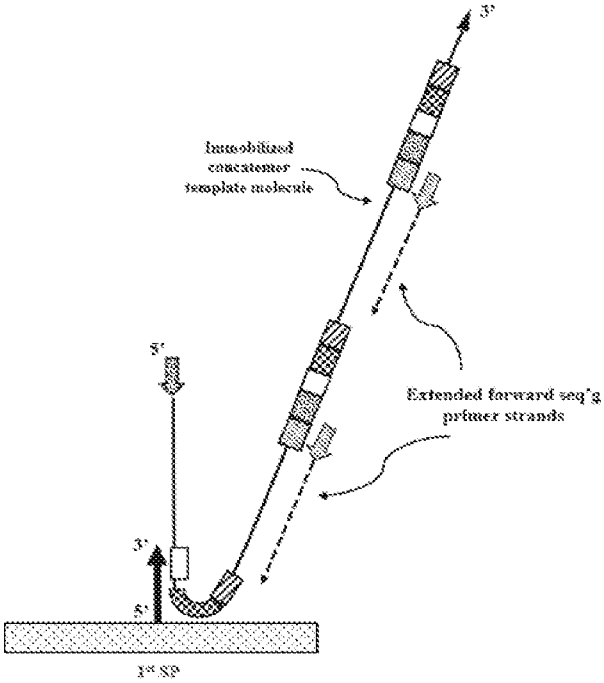


FIG. 99

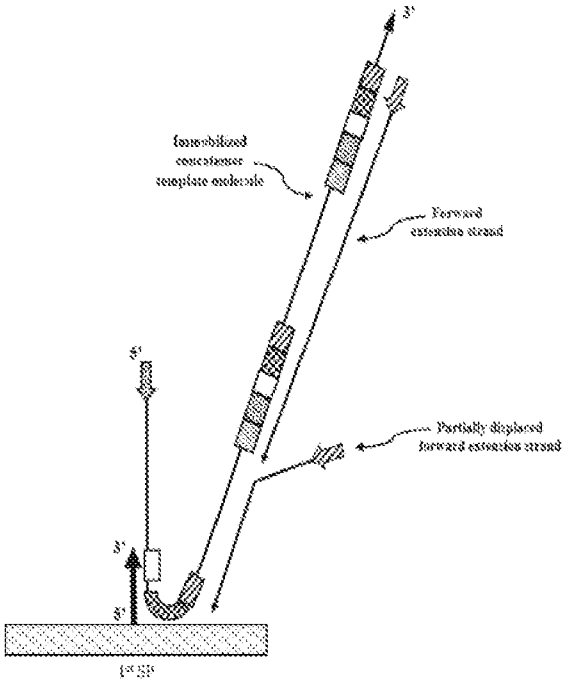


FIG. 100

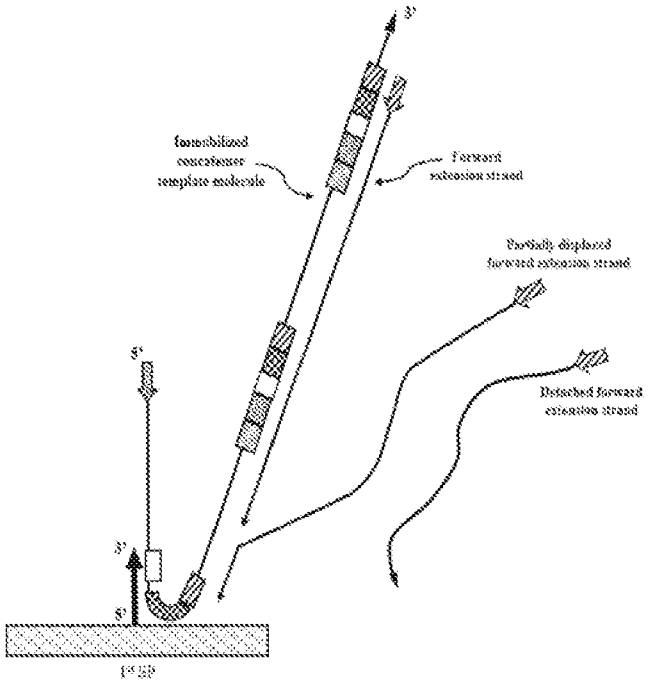


FIG. 101

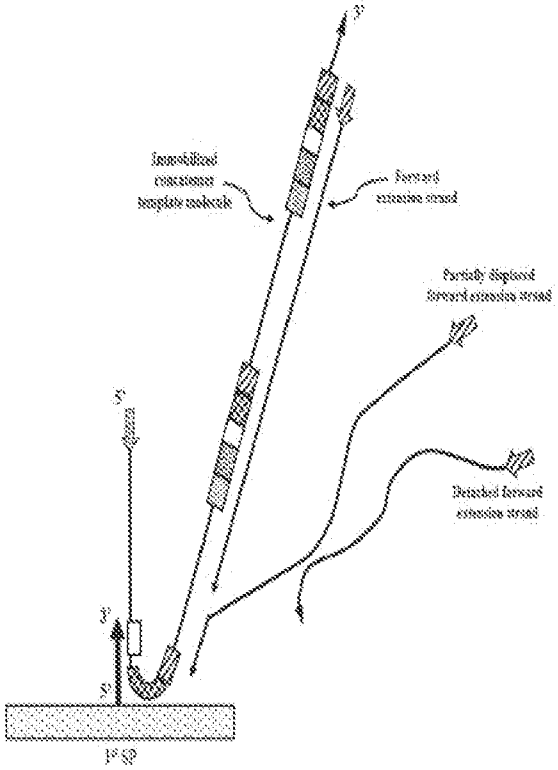


FIG. 102

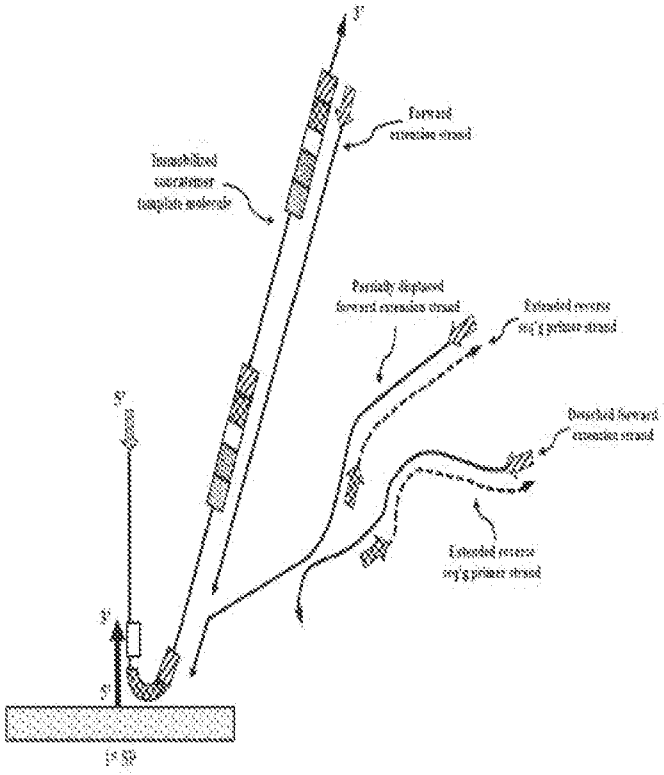


FIG. 103

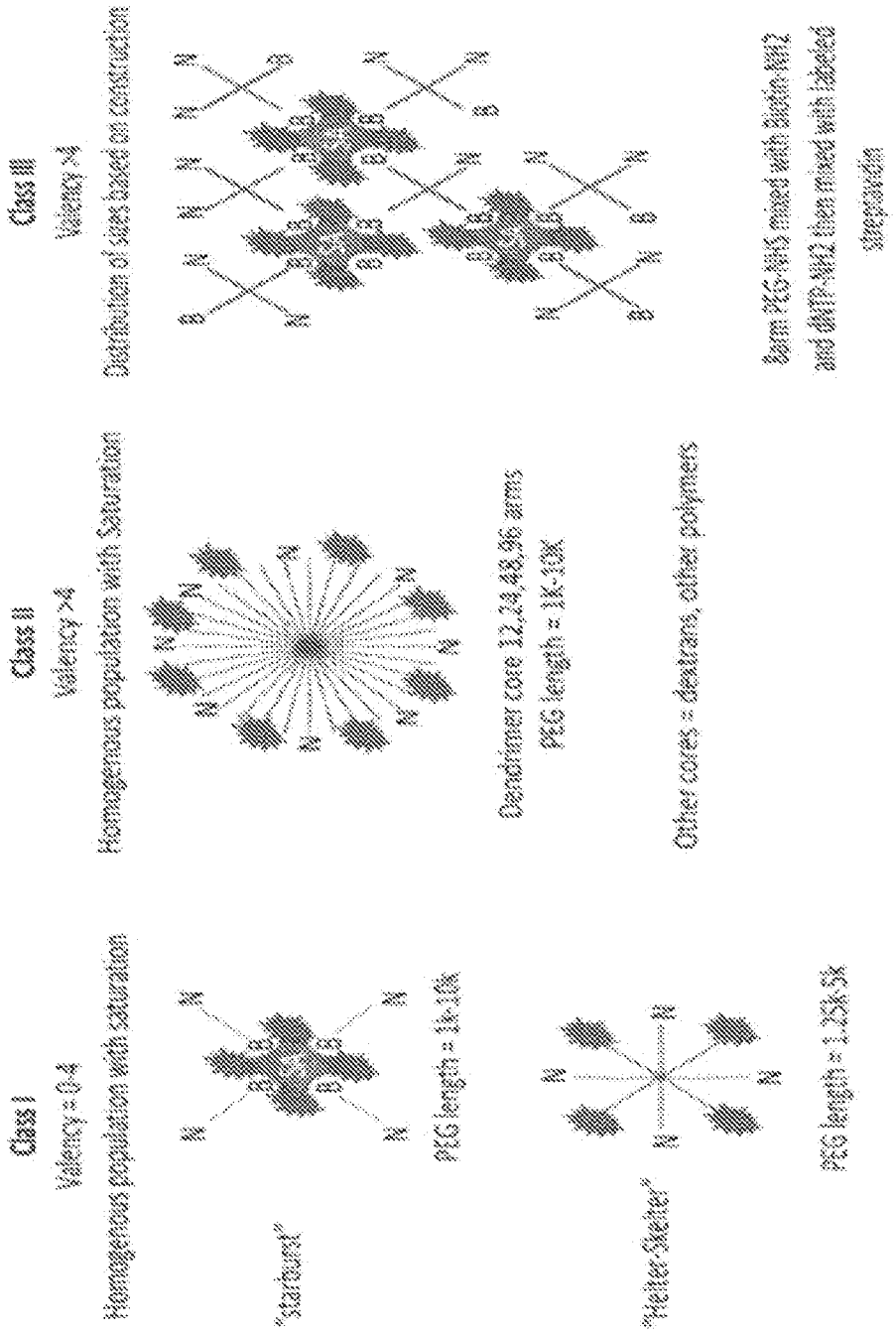

Class I
Valency = 0-4
Homogeneous population with saturation
"starburst"
PEG length = 1k-10k
"Heller-Skeiter"
PEG length = 1.25k-5k
Class II
Valency >4
Homogeneous population with Saturation
Dendrimer core 12,24,48,96 arms
PEG length = 1K-10K
Other cores = dextrans, other polymers
Class III
Valency >4
Distribution of ratio based on construction
Bare PEG-NH2 mixed with Biotin-NH2
and DMP-NH2 then mixed with labeled
streptavidin

FIG. 104

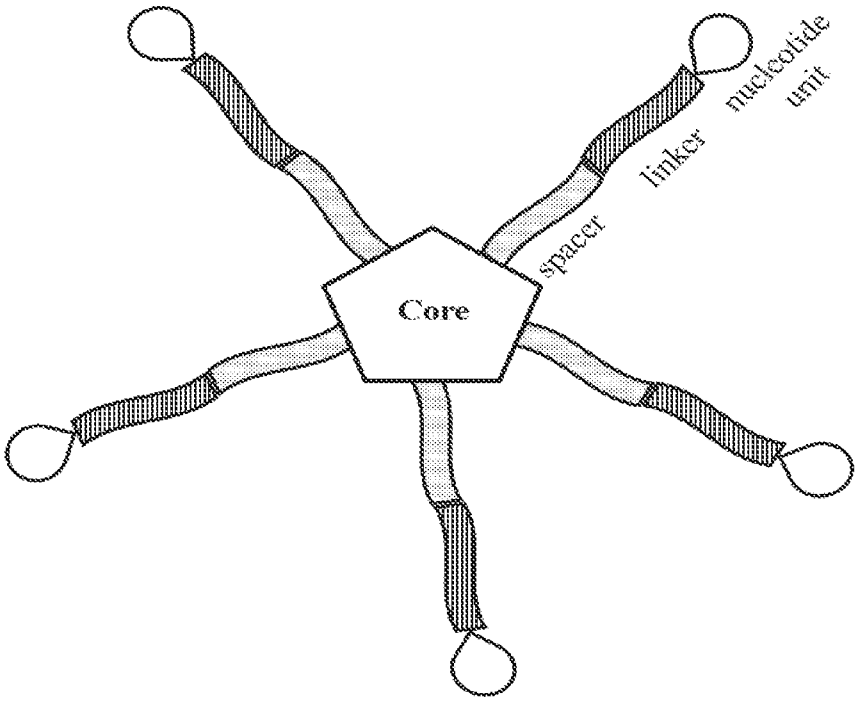


FIG. 105

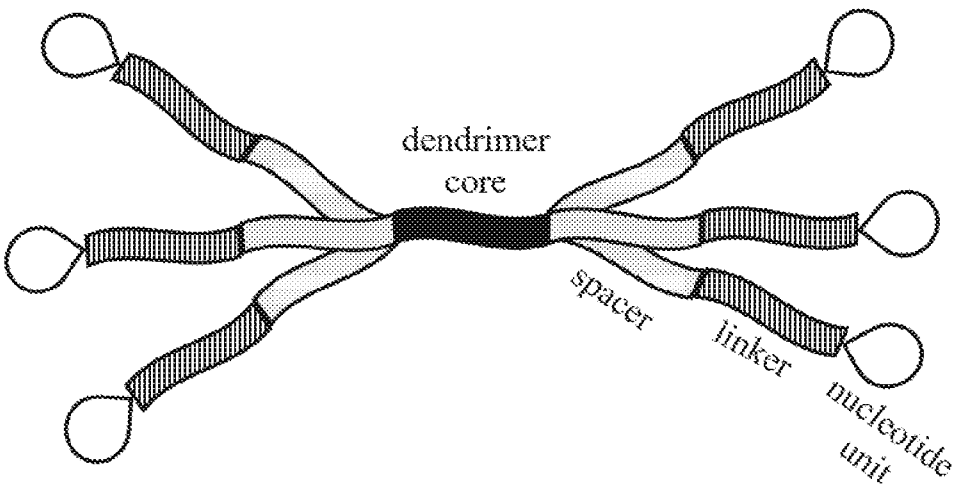


FIG. 106

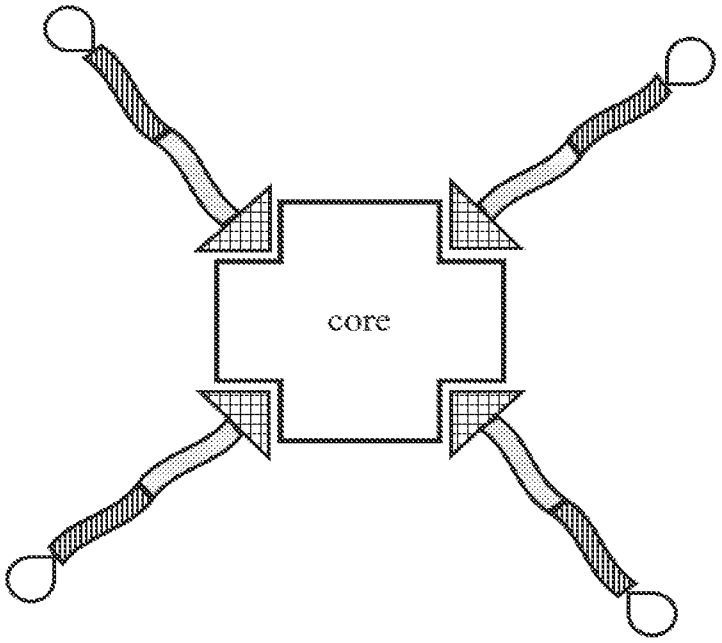


FIG. 107

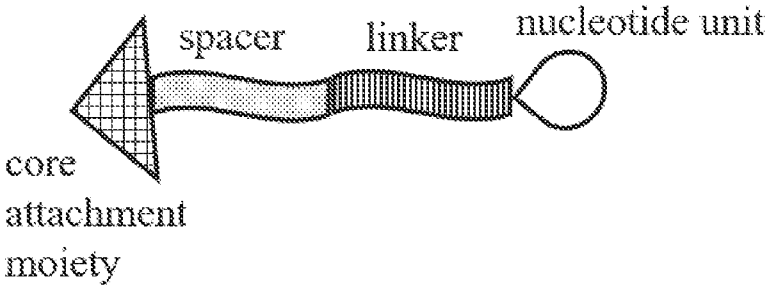
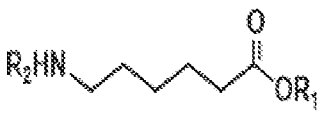


FIG. 108

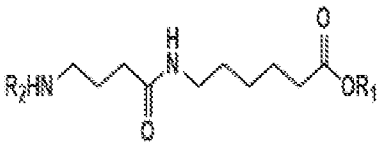
Spacer:

Linkers:

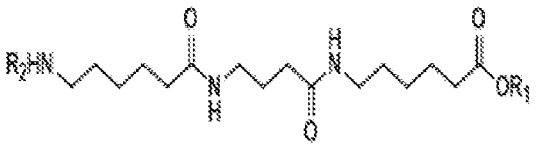
11 atom Linker:



16 atom Linker:



23 atom Linker:



N3 Linker:

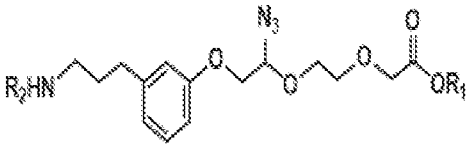
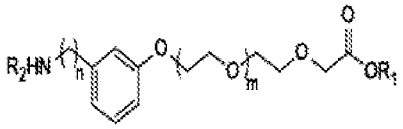
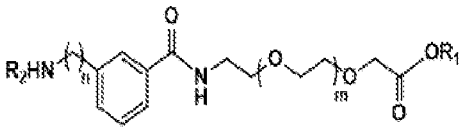


FIG. 109

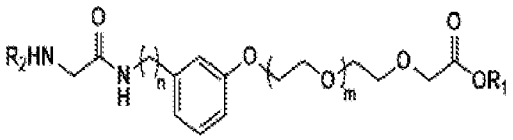
Linker 1:



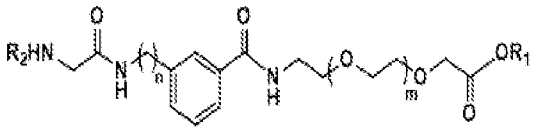
Linker 2:



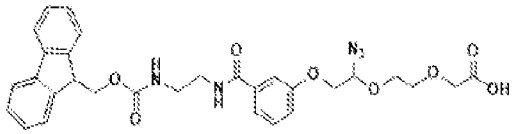
Linker 3:



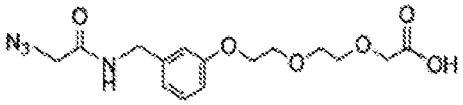
Linker 4:



Linker 5:

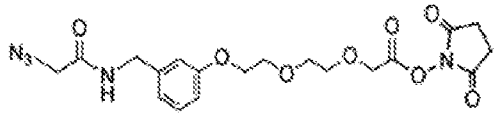


Linker 6:



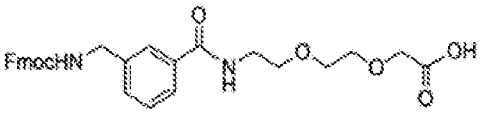
Molecular Weight: 352.35

Linker 7:



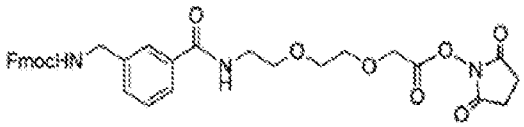
Molecular Weight: 449.42

Linker 8:



Molecular Weight: 518.57

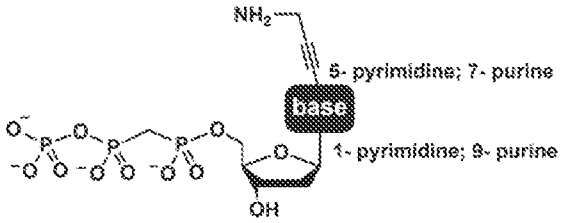
Linker 9:



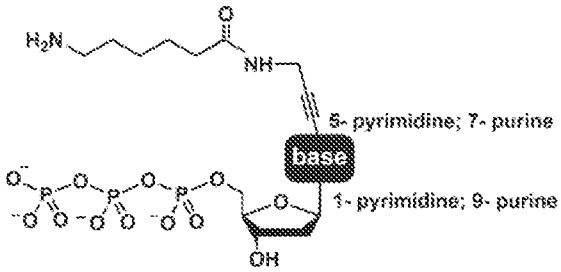
Molecular Weight: 615.64

FIG. 110

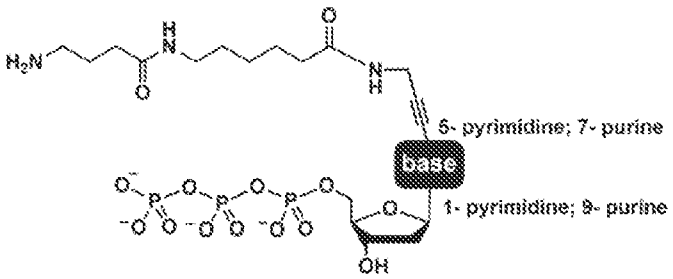
dNTP-PA-NH_2:



dNTP-PA-11 Atom Linker-NH_2:



dNTP-PA-16 Atom Linker-NH_2:



dNTP-PA-23 Atom Linker-NH_2:

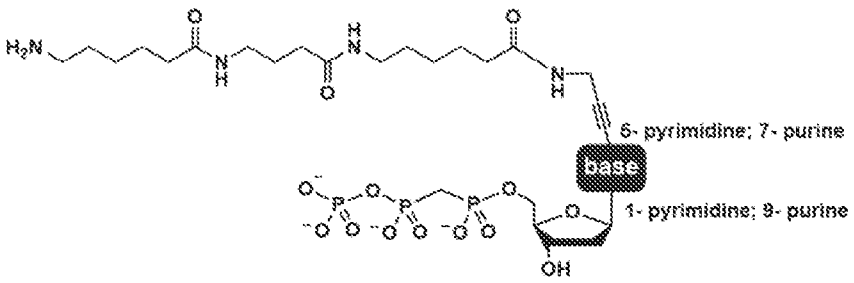
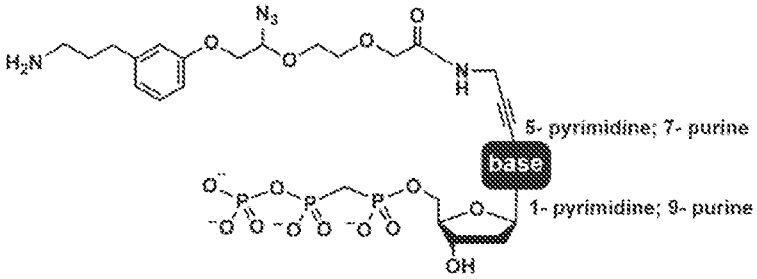
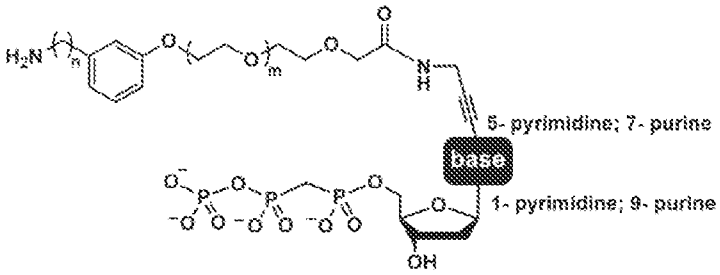


FIG. 111

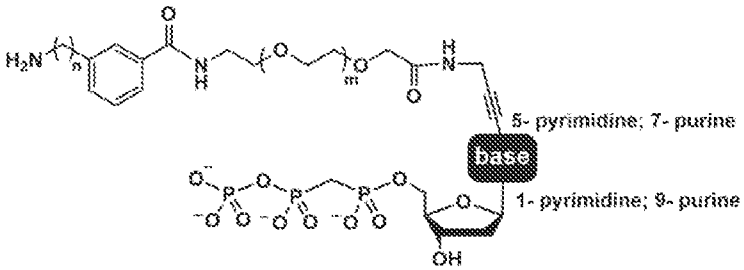
dNTP-PA-N3 Linker-NH_2:



dNTP-PA-Linker 1-NH_2:



dNTP-PA-Linker 2-NH_2:



dNTP-PA-Linker 3-NH_2:

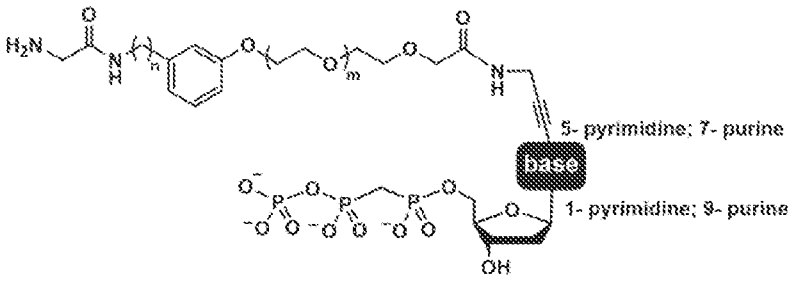
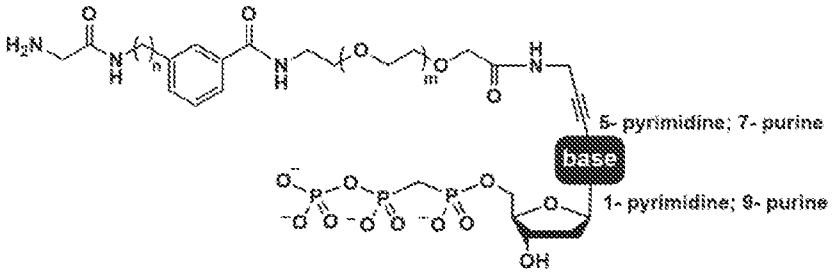


FIG. 112

dNTP-PA-Linker 4-NH_2:



dNTP-PA-N3 Linker-NH_2:

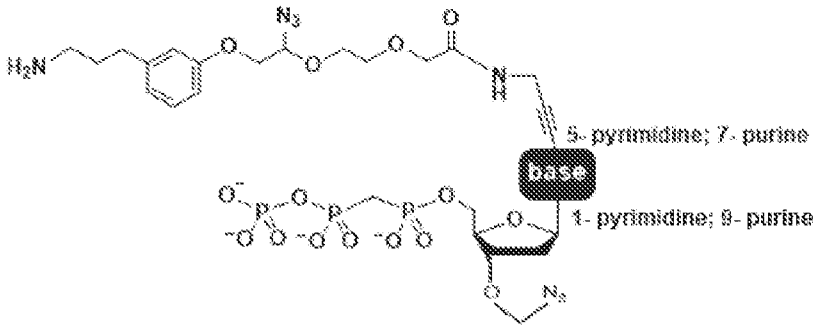


FIG. 113

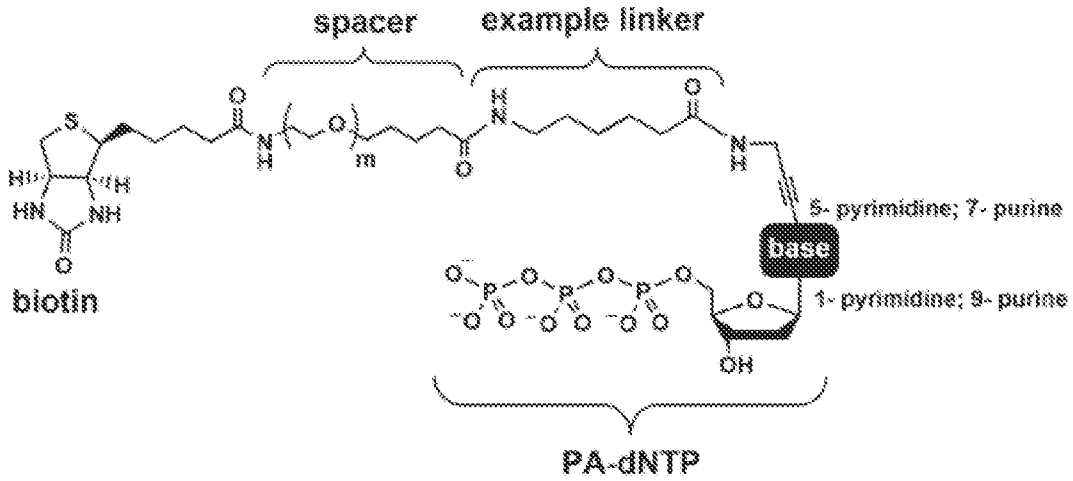


FIG. 114

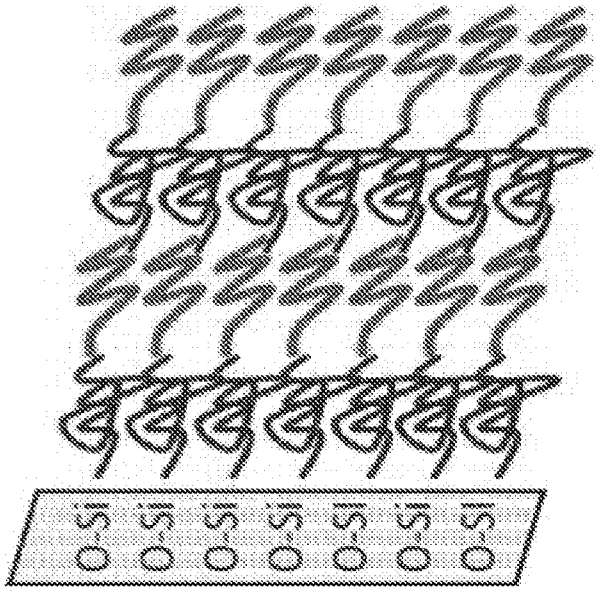
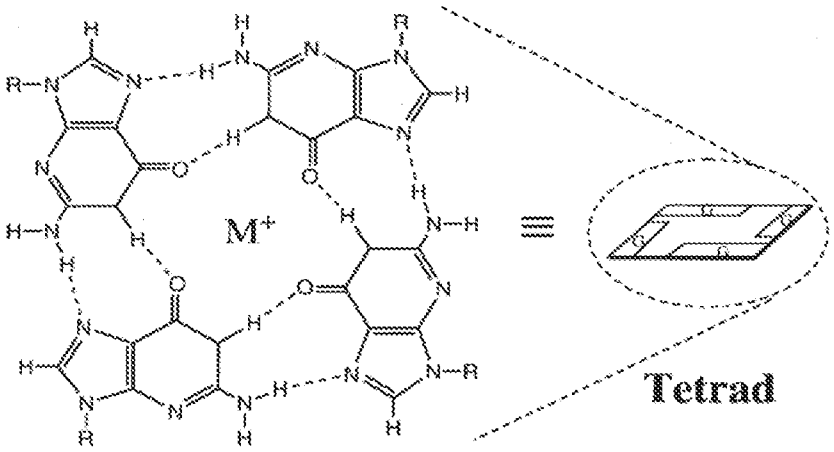


FIG. 115



G-tetrad

FIG. 116A

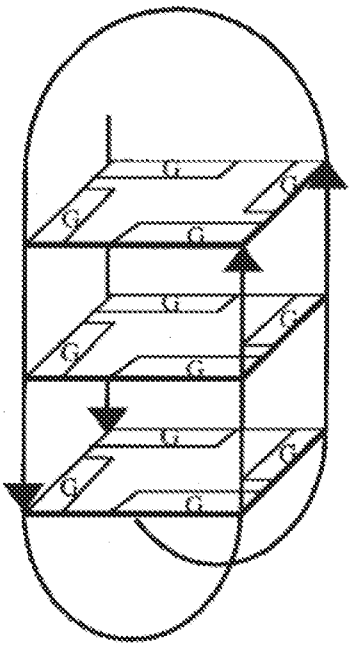


FIG. 116B

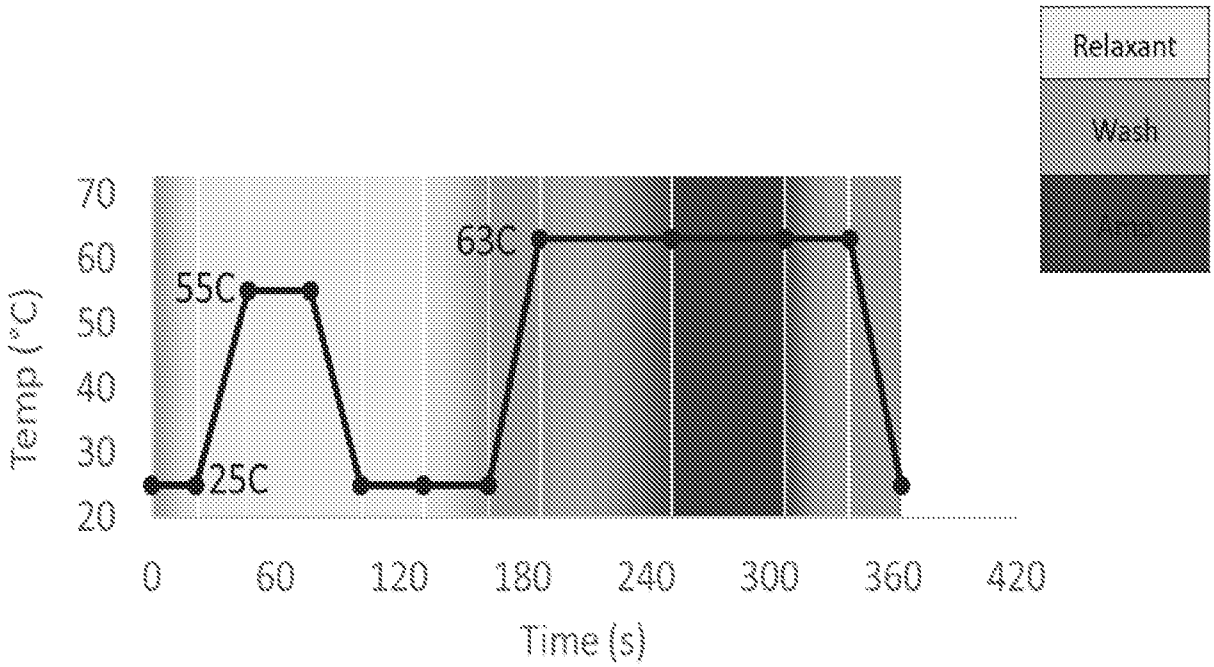

Relaxant
Wash
70
60
50
40
30
20
Temp (°C) duri
55C
63C
25C
0
60
120
180
240
300
360
420
Time (s)

FIG. 117

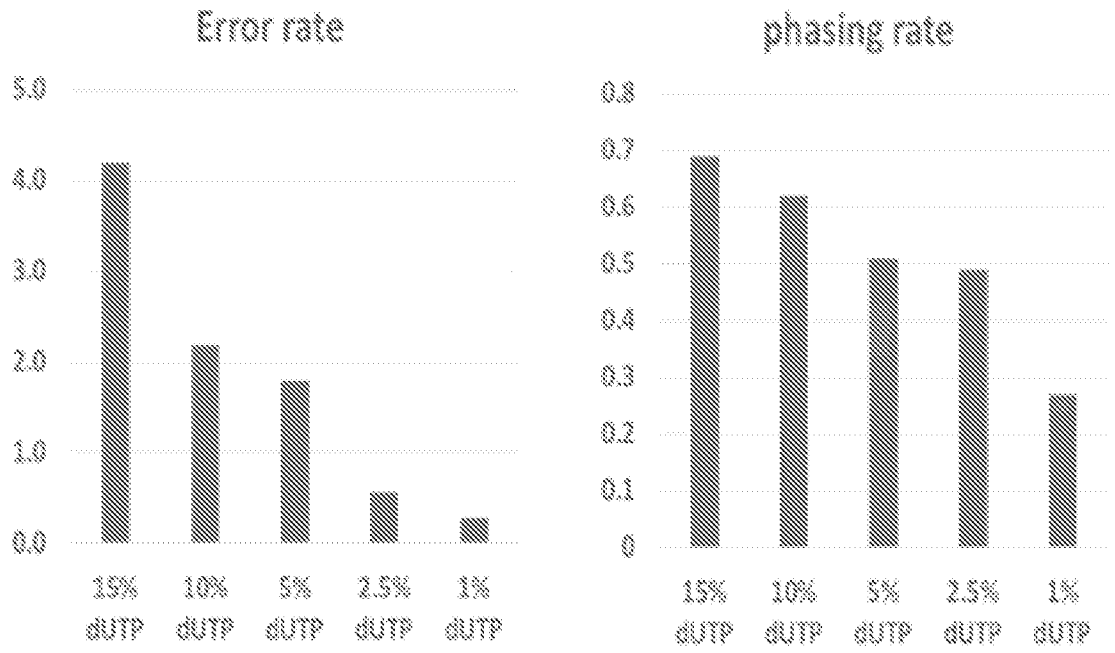

Error rate
5.0
4.0
3.0
2.0
1.0
0.0
15% dUTP
10% dUTP
5% dUTP
2.5% dUTP
1% dUTP
phasing rate
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
15% dUTP
10% dUTP
5% dUTP
2.5% dUTP
1% dUTP

FIG. 118

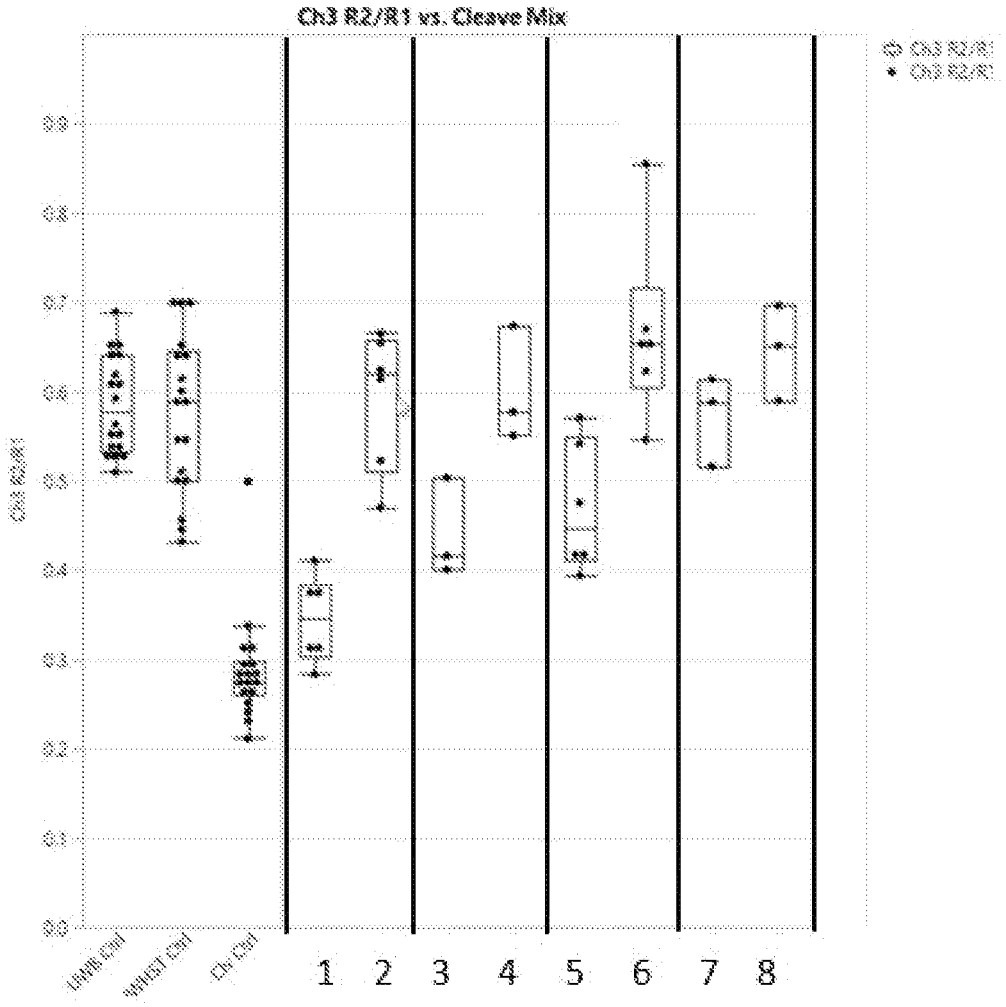


FIG. 119

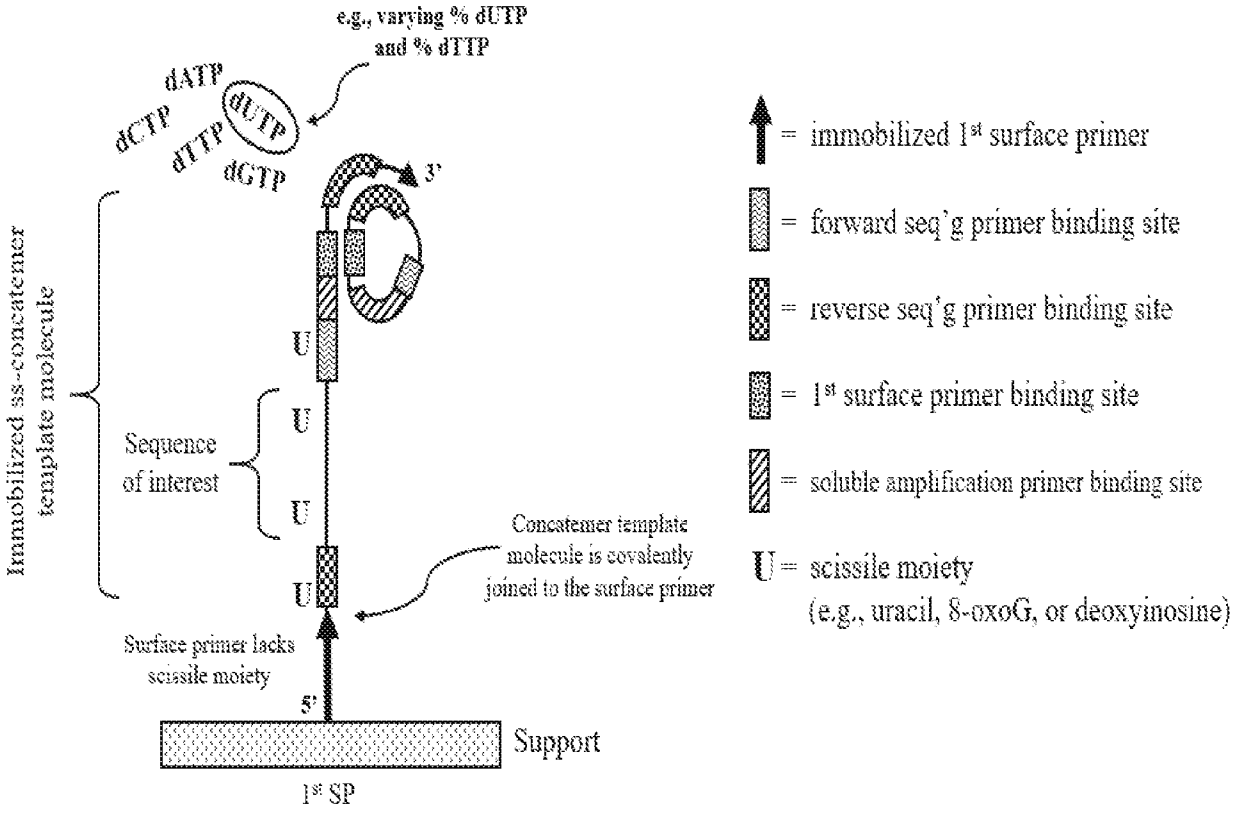

e.g., varying % dUTP
and % dTTP
dATP
dCTP
dTTP
dGTP
dUTP
3'
Immobilized ss-concatemer
template molecule
U
U
U
U
Sequence
of interest
Concatemer template
molecule is covalently
joined to the surface primer
Surface primer lacks
scissile moiety
5'
Support
1st SP
= immobilized 1st surface primer
= forward seq'g primer binding site
= reverse seq'g primer binding site
= 1st surface primer binding site
= soluble amplification primer binding site
U = scissile moiety
(e.g., uracil, 8-oxoG, or deoxyinosine)

FIG. 26