

SUMMARY

[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.

[0006a] In one aspect, the present disclosure refers to 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 individual concatemer template molecules, wherein individual concatemer template molecules in the plurality comprise two or more copies of a sequence of interest and binding sequences for a forward sequencing primer, a reverse sequencing primer, and a surface primer, and are covalently joined or hybridized to surface primers immobilized to a support, wherein the immobilized surface primers lack a nucleotide having a scissile moiety; 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 thereby generating retained 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, and wherein the linker is attached to the nucleotide unit.

[0007] In some examples, the individual concatemer template molecules in the plurality are covalently joined to an immobilized first surface primer. In some examples, the individual concatemer template molecules in the plurality are hybridized to an immobilized first surface primer. In some examples, 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 examples, 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 examples, 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 examples, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some examples, 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 examples, the plurality of immobilized second surface primers have 3' OH extendible ends. In some examples, the plurality of immobilized second surface primers have 3' non-extendible ends. In some examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some examples, 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 examples, the plurality of immobilized second surface primers have 3' OH extendible ends. In some examples, the plurality of immobilized second surface primers have 3' non-extendible ends. In some examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some examples, 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 examples, the plurality of immobilized second surface primers have 3' OH extendible ends. In some examples, the plurality of immobilized second surface primers have 3' non-extendible ends. In some examples, 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

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

[0021] In some examples, 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 examples, 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 examples, 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 examples, 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 examples, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some examples, 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 examples, the plurality of immobilized second surface primers have 3' OH extendible ends. In some examples, the plurality of immobilized second surface primers have 3' non-extendible ends. In some examples, the 3' non-extendible end comprises a phosphate group, a dideoxycytidine group, an inverted dT, or an amino group.

[0025] In some examples, 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 examples, 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 examples, individual concatemer template molecules in the plurality are covalently joined to an immobilized first surface primer. In some examples, individual concatemer template molecules in the plurality are hybridized to an immobilized first surface primer. In some examples, 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 examples, 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 examples, 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 examples, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some examples, 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 examples, the plurality of immobilized second surface primers have 3' OH extendible ends. In some examples, the plurality of immobilized second surface primers have 3' non-extendible ends. In some examples, 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.

[0030a] In one aspect, the present disclosure refers to 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 comprise two or more copies of a sequence of interest and binding sequences for a forward sequencing primer, a reverse sequencing primer, and a surface primer, and 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).

[0031] In some examples, 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 examples, 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 examples, 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 examples, 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 examples, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some examples, 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 examples, the plurality of immobilized second surface primers have 3' OH extendible ends. In some examples, the plurality of immobilized second surface primers have 3' non-extendible ends. In some examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, the support further comprises a plurality of immobilized second surface primers that lack a nucleotide having a scissile moiety. In some examples, 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 examples, the plurality of immobilized second surface primers have 3' OH extendible ends. In some examples, the plurality of immobilized second surface primers have 3' non-extendible ends. In some examples, 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

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 examples, individual concatemer template molecules in the plurality are covalently joined to an immobilized first surface primer. In some examples, individual concatemer template molecules in the plurality are hybridized to an immobilized first surface primer. In some examples, 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

(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 examples, 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 examples, 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.

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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, the immobilized concatemer template molecules comprise at least one nucleotide having a scissile moiety which comprises uridine, 8-oxo-7,8-dihydroguanine, or deoxyinosine.

[0053] In any of the foregoing or related examples, 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 examples, 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 examples, 0.01 – 30% of the guanosine nucleotides in the individual immobilized concatemer template molecules are replaced with 8-oxo-7,8-dihydroguanine or deoxyinosine.

[0055] In any of the foregoing or related examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 nucleobase of the incorporated nucleotide.

[0061] In some examples, 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 examples, 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 examples, 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 nucleobase 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 examples, 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 examples, 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 nucleobases of the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

[0066] In some examples, 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 examples, (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 examples, 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 examples, 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 examples, (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 examples, (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 examples, 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 nucleobase 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 examples, 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 examples, 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 nucleobases of the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

[0075] In some examples, 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 examples, (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 examples, (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 examples, (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 examples, 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 examples, (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 examples, (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 examples, (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 examples, 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 examples, the plurality of nucleotides comprises one type of nucleotide selected from a group consisting of dATP, dGTP, dCTP and dTTP. In some examples, 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 examples, at least one of the nucleotides in the plurality of nucleotides comprises a fluorescently-labeled nucleotide. In some examples, at least one of the plurality of nucleotides lacks a fluorophore label.

[0080] In any of the foregoing or related examples, 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 examples, 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-TTP), or Tri(hydroxypropyl)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 examples, 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 examples, (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(hydroxypropyl)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 examples, 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 examples, the core comprises an avidin-type moiety and the core attachment moiety comprises biotin. In some examples, the linker comprises an aliphatic chain having 2-6 subunits or an oligo ethylene glycol chain having 2-6 subunits. In some examples, the linker further comprises an aromatic moiety. In some examples, the nucleotide unit comprises an aromatic base, a five carbon sugar and 1-10 phosphate groups. In some examples, the linker is attached to the nucleotide unit through the base.

[0085] In some examples, 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 examples, 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 examples, 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 examples, the plurality of multivalent molecules are fluorescently-labeled multivalent molecules. In some examples, (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 examples, the plurality of multivalent molecules lack a fluorophore.

[0088] In some examples, 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 examples, (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(hydroxypropyl)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 examples, 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 examples, (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(hydroxypropyl)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 examples, 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 examples, 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 examples, the plurality of first sequencing polymerases of step (a) comprise a recombinant wild type DNA polymerase. In some examples, the plurality of first sequencing polymerases of step (a) comprise mutant DNA polymerase.

[0095] In some examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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