

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 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 or (ix) two or more copies of a unique molecular index sequence, 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 at least one nucleotide 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

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

What is claimed:

1. A method for pairwise sequencing, comprising:

a) providing a plurality of immobilized single stranded nucleic acid concatemer template molecules each comprising at least one nucleotide having a scissile moiety that can be cleaved to generate an abasic site in individual concatemer template molecules, wherein individual concatemer template molecules in the plurality comprise two or more copies of a sequence of interest and 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 or (ix) two or more copies of a unique molecular index sequence, 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 at least one nucleotide 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.

2. The method of claim 1, wherein:

a) a nucleotide unit of an individual multivalent molecule of step (b) binds a first polymerase which is bound to a nucleic acid duplex comprising an immobilized concatemer template molecule hybridized to a forward sequencing primer;

b) a nucleotide unit of an individual multivalent molecule of step (e) binds a second polymerase which is bound to a nucleic acid duplex comprising a retained forward extension strand hybridized to a reverse sequencing primer;

c) the core comprises streptavidin and the core attachment moiety comprises biotin;

d) the spacer comprises a polyethylene glycol (PEG) moiety, and wherein the linker comprises an aliphatic chain having 2-6 subunits or an oligo ethylene glycol chain having 2-6 subunits; or

e) the plurality of nucleotide arms attached to the core has the same type of a nucleotide unit, and wherein the type of nucleotide unit is selected from the group consisting of dATP, dGTP, dCTP, dTTP, and dUTP.

3. The method of claim 1, wherein:

a) the first plurality of multivalent molecules of step (b) and the second plurality of multivalent molecules of step (e) comprises one type of a multivalent molecule wherein each multivalent molecule in the plurality has the same type of nucleotide unit selected from the group consisting of dATP, dGTP, dCTP, dTTP, and dUTP, or

b) the first plurality of multivalent molecules of step (b) and the second plurality of multivalent molecules of step (e) comprises a mixture of any combination of two or more types of multivalent molecules, each type having nucleotide units selected from the group consisting of dATP, dGTP, dCTP, dTTP, and dUTP.

4. The method of claim 1, wherein at least one multivalent molecule in the first plurality of multivalent molecules of step (b) is labeled with a fluorophore, and wherein at least one multivalent molecule in the second plurality of multivalent molecules of step (e) is labeled with a fluorophore.

5. The method of claim 1, wherein the at least one nucleotide having a scissile moiety comprises a uridine, wherein the generating the abasic sites at the uridines at step (d) comprises contacting the immobilized concatemer template molecules with uracil DNA glycosylase (UDG), or wherein generating the plurality of gap-containing single stranded

nucleic acid concatemer template molecules of step (d) comprises contacting the retained immobilized template molecules containing one or more abasic sites with an endonuclease IV, AP lyase, FPG glycosylase/AP lyase, and/or endo VIII glycosylase/AP lyase, optionally wherein the AP lyase comprises DNA-apurinic lyase or DNA-apyrimidinic lyase.

6. The method of claim 1, wherein the support comprises at least one hydrophilic polymer coating layer and a plurality of surface primers immobilized to the at least one hydrophilic polymer coating layer, and wherein the at least one hydrophilic polymer coating layer has a water contact angle of no more than 45 degrees.

7. The method of claim 6, wherein the at least one hydrophilic polymer coating layer comprises a molecule selected from the group consisting of polyethylene glycol (PEG), poly(vinyl alcohol) (PVA), poly(vinyl pyridine), poly(vinyl pyrrolidone) (PVP), poly(acrylic acid) (PAA), polyacrylamide, poly(N-isopropylacrylamide) (PNIPAM), poly(methyl methacrylate) (PMA), poly(2-hydroxyethyl methacrylate) (HEMA), poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), polyglutamic acid (PGA), poly-lysine, polyglucoside, streptavidin, and dextran,

wherein the at least one hydrophilic polymer coating layer comprises polyethylene glycol (PEG),

wherein the at least one hydrophilic polymer coating layer comprises polymer molecules having a molecular weight of at least 1000 Daltons,

wherein the at least one hydrophilic polymer coating layer comprises branched polymer molecules having 4-8 branches, or

wherein the support comprises one hydrophilic polymer coating layer and a plurality of surface primers at a surface density of least 1000/ μm^2 .

8. The method of claim 6, wherein the support comprises:

- a) a first coating layer comprising a first monolayer of hydrophilic polymer molecules tethered to the support;
- b) a second coating layer comprising a second monolayer of hydrophilic polymer molecules tethered to the first monolayer; and
- c) a third coating layer comprising a third monolayer of hydrophilic polymer molecules tethered to the second monolayer, and wherein the hydrophilic polymer molecules of the first monolayer, second monolayer or third monolayer comprise branched polymer layers, or
 - (i) wherein the surface primers are immobilized to the hydrophilic polymer molecules of the second monolayer or third monolayer, and the surface primers are distributed at a plurality of depths throughout the second monolayer or the third monolayer,

(ii) wherein one or more of the at least one hydrophilic polymer coating layers comprise a plurality of surface primers at a surface density of least $1000/\mu\text{m}^2$, or

(iii) wherein one or more of the at least one hydrophilic polymer coating layers comprise a plurality of surface primers at a surface density of 1,000 – 15,000 per μm^2 .

9. The method of claim 6, wherein the at least one hydrophilic polymer coating layer on the support exhibits a contrast-to-noise (CNR) ratio of at least 20 when a fluorescent image of the support is obtained by contacting the support with a fluorescently-labeled nucleotide (Cy3-labeled nucleotide) and acquiring a fluorescence image using an inverted fluorescence microscope and a camera under non-signal saturating conditions while the support is immersed in a buffer,

wherein the support comprises glass or plastic, or

wherein the support is configured on a flowcell, or an interior of a capillary lumen.

10. The method of claim 1, wherein at least one of the immobilized concatemer template molecules in the plurality comprises a uridine-containing concatemer template molecule having up to 30% of thymidines replaced with uridine; and

wherein step c) comprises removing the plurality of extended forward sequencing primer strands while retaining the immobilized concatemer template molecules, and conducting a primer extension reaction with a plurality of soluble extension primers, a plurality of nucleotides and a plurality of primer extension polymerases, thereby generating the plurality of forward extension strands that are hybridized to the retained immobilized concatemer template molecules.

11. The method of claim 1, wherein at least one immobilized concatemer template molecule of the plurality of immobilized concatemer template molecules lacks a uridine,

a) wherein the plurality of soluble extension primers comprise a plurality of soluble amplification primers that hybridize to the universal binding sequence for the soluble amplification primer, or

b) wherein the plurality of soluble extension primers comprise a plurality of soluble forward sequencing primers that hybridize to the universal binding sequence for the soluble forward sequencing primer.

12. The method of claim 1, wherein the nucleotide unit of step (b) comprises a removable chain terminating moiety at the 3' sugar group, and wherein the nucleotide unit of step (e) comprises a removable chain terminating moiety at the 3' sugar group, wherein the removable chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, azido group, O-azidomethyl group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide

group, carbonate group, urea group, or silyl group, and wherein the removable chain terminating moiety is cleavable with a chemical compound to generate an extendible 3'OH moiety on the sugar group, or

wherein the sequencing of step (b) or step (e) comprises:

a₁) contacting the plurality of a first sequencing polymerases to (i) the plurality of immobilized concatemer template molecules or the plurality of retained forward extension strands, and (ii) the plurality of soluble sequencing primers which comprise a plurality of soluble forward sequencing primers or a plurality of soluble reverse sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each comprising a first sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises a nucleic acid template molecule hybridized to a soluble sequencing primer;

b₁) contacting the plurality of first complexed polymerases with a plurality of fluorophore-labeled multivalent molecules to form a plurality of multivalent-complexed polymerases, wherein the contacting is conducted under a condition suitable for binding complementary nucleotide units of the multivalent molecules to at least two of the plurality of first complexed polymerases thereby forming a plurality of multivalent-complexed polymerases, and the condition inhibits incorporation of the complementary nucleotide units into the hybridized sequencing primers of the plurality of multivalent-complexed polymerases;

c₁) detecting the plurality of multivalent-complexed polymerases; and

d₁) 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, and optionally

further comprising:

e₁) dissociating the plurality of multivalent-complexed polymerases by removing the plurality of first sequencing polymerases and their bound multivalent molecules, and retaining the plurality of nucleic acid duplexes;

f₁) contacting the plurality of the retained nucleic acid duplexes of step (e₁) with a plurality of a second sequencing polymerase, wherein the contacting is conducted under a condition suitable for binding the plurality of second sequencing polymerases to the plurality of the retained nucleic acid duplexes, thereby forming a plurality of second complexed polymerases each comprising a second sequencing polymerase bound to a retained nucleic acid duplex; and

g₁) contacting the plurality of second complexed polymerases with a plurality of nucleotides, wherein the contacting is conducted under a condition suitable for binding complementary nucleotides to at least two of the second complexed polymerases of step (f₁) thereby forming a plurality of nucleotide-complexed polymerases and the condition is suitable for promoting incorporation of the bound complementary nucleotides into the hybridized sequencing primers of the nucleotide-complexed polymerases, thereby generating a plurality of extended sequencing primer strands, wherein the plurality of extended sequencing primer strands comprise a plurality of extended forward sequencing primer strands or a plurality of extended reverse sequencing primer strands.

13. The method of claim 10, wherein the generating the abasic sites at the uridines of the immobilized concatemer template molecules of step (d) comprises contacting the immobilized concatemer template molecule with uracil DNA glycosylase (UDG), or

wherein generating the plurality of gap-containing concatemer template molecules of step (d) comprises contacting the retained immobilized template molecules containing one or more abasic sites with an endonuclease IV, AP lyase, FPG glycosylase/AP lyase and/or endo VIII glycosylase/AP lyase, optionally wherein the AP lyase comprises DNA-apurinic lyase or DNA-apyrimidinic lyase.

14. The method of claim 10, wherein the support comprises:

- a) a first coating layer comprising a first monolayer of hydrophilic polymer molecules tethered to the support;
- b) a second coating layer comprising a second monolayer of hydrophilic polymer molecules tethered to the first monolayer; and
- c) a third coating layer comprising a third monolayer of hydrophilic polymer molecules tethered to the second monolayer, and wherein the hydrophilic polymer molecules of the first monolayer, second monolayer or third monolayer comprise branched polymer layers.

15. The method of claim 14, wherein the surface primers are immobilized to the hydrophilic polymer molecules of the second monolayer or the third monolayer, and the surface primers are distributed at a plurality of depths throughout the second monolayer or the third monolayer.

16. The method of claim 1, wherein the scissile moiety comprises uridine, 8-oxo-7,8-dihydroguanine or deoxyinosine.

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

18. The method of claim 17, wherein the support comprises at least one hydrophilic polymer coating layer and a plurality of surface primers immobilized to the at least one hydrophilic polymer coating layer, and wherein the at least one hydrophilic polymer coating layer has a water contact angle of no more than 45 degrees.

19. The method of claim 18, wherein the at least one hydrophilic polymer coating layer exhibits a contrast-to-noise (CNR) ratio of at least 20 when a fluorescent image of the support is obtained by contacting the support with a fluorescently-labeled nucleotide (Cy3-labeled nucleotide) and acquiring a fluorescence image using an inverted fluorescence microscope and a camera under non-signal saturating conditions while the support is immersed in a buffer.

20. The method of claim 17, wherein replacing the plurality of extended forward sequencing primer strands of step (d) comprises:

- (i) removing the plurality of extended forward sequencing primer strands while retaining the immobilized concatemer template molecules; and
- (ii) contacting the plurality of retained immobilized concatemer molecules with the plurality of soluble amplification primers, a plurality of nucleotides and a plurality of strand displacing polymerases, under a condition suitable to hybridize the plurality of soluble amplification primers to the plurality of retained immobilized concatemer template molecules and suitable for conducting polymerase-catalyzed strand displacing reactions thereby generating a plurality of forward extension strands and a plurality of partially displaced extended forward sequencing strands that are hybridized to the immobilized concatemer template molecules to form a plurality of immobilized amplicons, optionally

wherein the strand displacing polymerase comprises phi29 DNA polymerase, large fragment of Bst DNA polymerase, large fragment of Bsu DNA polymerase (exo-), Bca DNA polymerase (exo-), Klenow fragment of E. coli DNA polymerase, T5 polymerase, M-MuLV reverse transcriptase, HIV viral reverse transcriptase, Deep Vent DNA polymerase and KOD DNA polymerase.

21. The method of claim 17, wherein the sequencing of step (c) comprises:

a₁) contacting a plurality of sequencing polymerases and a plurality of the soluble forward sequencing primers to a plurality of immobilized concatemer template molecules, wherein the contacting is conducted under a condition suitable to form a plurality of complexed polymerases each comprising a sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises an immobilized concatemer template molecule hybridized to a soluble forward sequencing primer;

b₁) 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;

c₁) 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

d₁) detecting the incorporated nucleotide and identifying the nucleo-base of the incorporated nucleotide, optionally

wherein at least one of the nucleotides in the plurality of nucleotides of step (b₁) comprises a removable chain terminating moiety attached to the 3' carbon position of the sugar group, wherein the removable chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, azido group, O-azidomethyl group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group, and wherein

the removable chain terminating moiety is cleavable with a chemical compound to generate an extendible 3'OH moiety on the sugar group.

22. The method of claim 17, wherein the sequencing of step (e) comprises:

a₂) contacting a plurality of sequencing polymerases and a plurality of the soluble reverse sequencing primers to the plurality of the immobilized partially displaced forward extension strands, wherein the contacting is conducted under a condition suitable to form a plurality of complexed polymerases each comprising a sequencing polymerase bound to a nucleic acid duplex wherein the nucleic acid duplex comprises a soluble reverse sequencing primer hybridized to an immobilized partially displaced forward extension strand;

b₂) 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;

c₂) 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

d₂) detecting the incorporated nucleotide and identifying the nucleobase of the incorporated nucleotide, optionally

wherein at least one of the nucleotides in the plurality of nucleotides of step (b₂) comprises a removable chain terminating moiety attached to the 3' carbon position of the sugar group, wherein the removable chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, azido group, O-azidomethyl group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group, and wherein the removable chain terminating moiety is cleavable with a chemical compound to generate an extendible 3'OH moiety on the sugar group.

23. The method of claim 17, wherein the sequencing of step (c) and the sequencing of step (e) comprise:

a₃) contacting a plurality of a first sequencing polymerase and a plurality of soluble sequencing primers to a plurality of nucleic acid template molecules, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each comprising a first sequencing polymerase bound to a nucleic acid duplex which comprises the nucleic acid template molecule hybridized to the soluble sequencing primer, wherein (1) the plurality of nucleic acid template molecules comprise a plurality of the immobilized concatemer template molecules and the plurality of sequencing primers comprise a plurality of the soluble forward sequencing primers, or wherein (2) the plurality of nucleic acid template molecules comprise a plurality of immobilized partially displaced forward extension strands and the plurality of sequencing primers comprise a plurality of the soluble reverse sequencing primers;

b₃) contacting the plurality of first complexed polymerases with a plurality of detectably labeled multivalent molecules to form a plurality of multivalent-complexed polymerases, wherein individual multivalent molecules in the plurality of multivalent molecules comprise a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit, wherein the contacting is performed under a condition suitable for binding complementary nucleotide units of the multivalent molecules to at least two of the plurality of first complexed polymerases thereby forming a plurality of multivalent-complexed polymerases, and the condition inhibits incorporation of the complementary nucleotide units into the sequencing primers of the plurality of multivalent-complexed polymerases;

c₃) detecting the plurality of multivalent-complexed polymerases; and

d₃) identifying the nucleo-base of the complementary nucleotide units that are bound to the plurality of first complexed polymerases in the plurality of multivalent-complexed polymerases, thereby determining the sequence of the nucleic acid template, optionally

further comprising:

e₃) 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;

f₃) contacting the plurality of the retained nucleic acid duplexes of step (e₃) 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;

g₃) contacting the plurality of second complexed polymerases with a plurality of nucleotides under a condition suitable for binding complementary nucleotides from the plurality of nucleotides to at least two of the second complexed polymerases of step (f₃) 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, and optionally

further comprising:

h₃) detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases; and

i₃) identifying the nucleo-bases of the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

24. The method of claim 23, further comprising: forming at least one avidity complex in step (b), the method comprising:

a₄) 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

b₄) 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, optionally:

wherein (i) the first sequencing primer comprises a soluble forward sequencing primer and the nucleic acid template molecule comprises an immobilized concatemer template molecule, (ii) the second sequencing primer comprises a soluble forward sequencing primer and the nucleic acid template molecule comprises the same immobilized concatemer template molecule, and (iii) the first and second sequencing primers have the same sequence, or

wherein (i) the first sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises an immobilized partially displaced forward extension strand, (ii) the second sequencing primer comprises a soluble reverse sequencing primer and the nucleic acid template molecule comprises the same immobilized partially displaced forward extension strand, and (iii) the first and second sequencing primers have the same sequence.

25. The method of claim 23, wherein individual multivalent molecules in the plurality of multivalent molecules comprise: (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 the core attachment moiety, wherein the spacer is attached to the linker, and wherein the linker is attached to the nucleotide unit, optionally

wherein

- (i) the core comprises streptavidin and the core attachment moiety comprises biotin;
- (ii) the linker comprises an aliphatic chain having 2-6 subunits or an oligo ethylene glycol chain having 2-6 subunits; and
- (iii) the nucleotide unit comprises an aromatic base, a five carbon sugar and 1-10 phosphate groups, or

wherein the plurality of nucleotide arms attached to the core has the same type of nucleotide unit, and wherein the type of nucleotide unit is selected from the group consisting of dATP, dGTP, dCTP, dTTP, and dUTP, or

wherein the plurality of multivalent molecules comprises one type of multivalent molecule, wherein each multivalent molecule in the plurality has the same type of nucleotide unit selected from the group consisting of dATP, dGTP, dCTP, dTTP, and dUTP, or

wherein the plurality of multivalent molecules comprises a mixture of any combination of two or more types of multivalent molecules, each type having nucleotide units selected from the group consisting of dATP, dGTP, dCTP, dTTP, and dUTP.

26. The method of claim 23, wherein the plurality of detectably labeled multivalent molecules comprise fluorescently-labeled multivalent molecules.