

SUMMARY

[0004a] In one aspect, the present disclosure relates to a library-splint complex (300) comprising:

- (I) a single-stranded nucleic acid library molecule (100) comprising components arranged in a 5' to 3' order: (i) a first left universal adaptor sequence (120) having a binding sequence for a first surface primer immobilized to a support; (ii) a second left universal adaptor sequence (140) having a binding sequence for a first sequencing primer; (iii) a sequence of interest (110); (iv) a second right universal adaptor sequence (150) having a binding sequence for a second sequencing primer; and (v) a first right universal adaptor sequence (130) having a binding sequence for a second surface primer immobilized to the support, wherein the single-stranded nucleic acid library molecule further comprises a first left unique identification sequence (180) and/or a first right unique identification sequence (190); and
- (II) a single-stranded splint strand (200) comprising, in a 5' to 3' order: (i) a first region (210) having a universal binding sequence for the first left universal adaptor sequence (120) of the single-stranded nucleic acid library molecule (100), and (ii) a second region (220) having a universal binding sequence for the first right universal adaptor sequence (130) of the single-stranded nucleic acid library molecule (100), wherein hybridization of the single-stranded splint strand (200) to the single-stranded nucleic acid library molecule (100) generates a library-splint complex (300) having a nick between a 5' end of the library molecule and a 3' end of the library molecule.

[0004b] In another aspect, the present disclosure relates to a method for generating a plurality of covalently closed circular library molecules (400) comprising:

- a) providing a plurality of single-stranded nucleic acid library molecules (100), wherein individual library molecules in the plurality comprise regions arranged in a 5' to 3' order: (i) a first left universal adaptor sequence (120) having a binding sequence for a first surface primer immobilized to a support; (ii) a second left universal adaptor sequence (140) having a binding sequence for a first sequencing primer; (iii) a sequence of interest (110); (iv) a second right universal adaptor sequence (150) having a binding sequence for a second sequencing primer; and (v) a first right universal adaptor sequence (130) having a binding sequence for a second surface primer immobilized to the support, wherein individual library molecules further comprise a first left unique identification sequence (180) and/or a first right unique identification sequence (190);

- b) providing a plurality of single-stranded splint strands (200), wherein individual single-stranded splint strands (200) in the plurality comprise, in a 5' to 3' order: (i) a first region (210) that hybridizes with the first left universal adaptor sequence (120) of individual library molecules, and (ii) a second region (220) that hybridizes with the first right universal adaptor sequence (130) of individual library molecules;
- c) hybridizing the plurality of single-stranded splint strands (200) with the plurality of single-stranded nucleic acid library molecules (100), wherein the first region of the individual single-stranded splint strands (210) anneals to the first left universal adaptor sequences (120) of individual library molecules and the second region of the individual single-stranded splint strands (220) anneals to the first right universal adaptor sequence (130) of individual library molecules, thereby circularizing individual single-stranded nucleic acid library molecules and forming a plurality of library-splint complexes (300) having a nick between the terminal 5' and 3' ends of individual library molecules; and
- d) ligating the nick in the plurality of library-splint complexes (300), thereby generating a plurality of covalently closed circular library molecules (400).

[0004c] In another aspect, the present disclosure relates to a method for generating a plurality of library-splint complexes (300) comprising:

- a) providing a plurality of single-stranded nucleic acid library molecules (100), wherein individual library molecules in the plurality comprise regions arranged in a 5' to 3' order: (i) a first left universal adaptor sequence (120) having a binding sequence for a first surface primer immobilized to a support; (ii) a second left universal adaptor sequence (140) having a binding sequence for a first sequencing primer; (iii) a sequence of interest (110); (iv) a second right universal adaptor sequence (150) having a binding sequence for a second sequencing primer; and (v) a first right universal adaptor sequence (130) having a binding sequence for a second surface primer immobilized to the support, wherein the plurality of single-stranded nucleic acid library molecules (100) further comprise a first left unique identification sequence (180) and/or a first right unique identification sequence (190);
- b) providing a plurality of single-stranded splint strands (200), wherein individual single-stranded splint strands (200) in the plurality comprise regions arranged in a 5' to 3' order: (i) a first region (210) having a universal binding sequence that hybridizes with the first left universal adaptor sequence (120) of a single-stranded nucleic acid library molecule (100), and (ii) a second region (220) having a universal binding sequence that hybridizes with the first right universal adaptor sequence (130) of the single-stranded nucleic acid library molecule (100);

c) hybridizing the plurality of single-stranded splint strands (200) with the plurality of single-stranded nucleic acid library molecules (100) under a condition suitable to hybridize the first region (210) of the single-stranded splint strand (200) to the first left universal adaptor sequence (120) of the linear single-stranded library molecule (100), and suitable to hybridize the second region (220) of the single-stranded splint strand (200) to first right universal adaptor sequence (130) of the linear single-stranded library molecule (100), thereby circularizing individual single-stranded nucleic acid library molecules to form a plurality of library-splint complexes (300) having a nick between the terminal 5' and 3' ends of a library molecule, wherein the nick is enzymatically ligatable.

[0005] The disclosure provides a library-splint complex (300) comprising: **(i)** a single-stranded nucleic acid library molecule (100) which includes a sequence of interest (110) flanked on one side by at least a first left universal adaptor sequence (120) and flanked on the other side by at least a first right universal adaptor sequence (130); and **(ii)** a single-stranded splint strand (200) which includes a first region (210) having a universal binding sequence for the first left universal adaptor sequence (120) of the library molecule, and the single-stranded splint strand further comprises a second region (220) having a universal binding sequence for the first right universal adaptor sequence (130) of the library molecule, thereby circularizing the library molecule to generate a library-splint complex (300) having a nick between the 5' end of the library molecule and the 3' end of the library molecule.

[0006] In some embodiments, the nucleic acid library molecule (100) further comprises: a second left universal adaptor sequence (140). In some embodiments, the nucleic acid library molecule (100) further comprises: a second right universal adaptor sequence (150). In some embodiments, the nucleic acid library molecule (100) further comprises: a first left sample index sequence (160). In some embodiments, the nucleic acid library molecule (100) further comprises: a first right sample index sequence (170). In some embodiments, the nucleic acid library molecule (100) further comprises: a first left unique identification sequence (180). In some embodiments, the nucleic acid library molecule (100) further comprises: a first right unique identification sequence (190).

[0007] In some embodiments, the nucleic acid library molecule (100) further comprises any one or any combination of two or more of: (i) a second left universal adaptor sequence (140); (ii) a second right universal adaptor sequence (150); (iii) a first left index sequence (160); (iv) a first right index sequence (170); (v) a first left unique identification sequence (180); and/or (vi) a first right unique identification sequence (190).

[0008] In some embodiments, the first left universal adaptor sequence (120) and/or the second left universal adaptor sequence (140), comprises: (i) a universal binding sequence for a forward sequencing primer; (ii) a universal binding sequence for a reverse sequencing primer; (iii) a universal binding sequence for an first surface primer; (iv) a universal binding sequence for an second surface primer; (v) a universal binding sequence for a forward amplification primer; (vi) a universal binding sequence for a reverse amplification primer; and/or (vii) a universal binding sequence for a compaction oligonucleotide.

WHAT IS CLAIMED:

1. A library-splint complex (300) comprising:
 - (I) a single-stranded nucleic acid library molecule (100) comprising components arranged in a 5' to 3' order: (i) a first left universal adaptor sequence (120) having a binding sequence for a first surface primer immobilized to a support; (ii) a second left universal adaptor sequence (140) having a binding sequence for a first sequencing primer; (iii) a sequence of interest (110); (iv) a second right universal adaptor sequence (150) having a binding sequence for a second sequencing primer; and (v) a first right universal adaptor sequence (130) having a binding sequence for a second surface primer immobilized to the support, wherein the single-stranded nucleic acid library molecule further comprises a first left unique identification sequence (180) and/or a first right unique identification sequence (190); and
 - (II) a single-stranded splint strand (200) comprising, in a 5' to 3' order: (i) a first region (210) having a universal binding sequence for the first left universal adaptor sequence (120) of the single-stranded nucleic acid library molecule (100), and (ii) a second region (220) having a universal binding sequence for the first right universal adaptor sequence (130) of the single-stranded nucleic acid library molecule (100), wherein hybridization of the single-stranded splint strand (200) to the single-stranded nucleic acid library molecule (100) generates a library-splint complex (300) having a nick between a 5' end of the library molecule and a 3' end of the library molecule.
2. The library-splint complex (300) of claim 1, wherein the single-stranded nucleic acid library molecule (100) further comprises any one or any combination of:
 - (vi) a first left sample index sequence (160), and/or
 - (vii) a first right sample index sequence (170).
3. The library-splint complex (300) of claim 1 or 2, wherein the first left universal adaptor sequence (120) and/or the second left universal adaptor sequence (140) comprises:
 - (i) a universal binding sequence for a forward sequencing primer;
 - (ii) a universal binding sequence for a reverse sequencing primer;
 - (iii) a universal binding sequence for a first surface primer;

- (iv) a universal binding sequence for a second surface primer;
- (v) a universal binding sequence for a forward amplification primer;
- (vi) a universal binding sequence for a reverse amplification primer; and/or
- (vii) a universal binding sequence for a compaction oligonucleotide.

4. The library-splint complex (300) of any one of claims 1-3, wherein the first right universal adaptor sequence (130) and/or the second right universal adaptor sequence (150) comprises:

- (i) a universal binding sequence for a forward sequencing primer;
- (ii) a universal binding sequence for a reverse sequencing primer;
- (iii) a universal binding sequence for a first surface primer;
- (iv) a universal binding sequence for a second surface primer;
- (v) a universal binding sequence for a forward amplification primer;
- (vi) a universal binding sequence for a reverse amplification primer; and/or
- (vii) a universal binding sequence for a compaction oligonucleotide.

5. A method for generating a plurality of covalently closed circular library molecules (400) comprising:

- a) providing a plurality of single-stranded nucleic acid library molecules (100), wherein individual library molecules in the plurality comprise regions arranged in a 5' to 3' order: (i) a first left universal adaptor sequence (120) having a binding sequence for a first surface primer immobilized to a support; (ii) a second left universal adaptor sequence (140) having a binding sequence for a first sequencing primer; (iii) a sequence of interest (110); (iv) a second right universal adaptor sequence (150) having a binding sequence for a second sequencing primer; and (v) a first right universal adaptor sequence (130) having a binding sequence for a second surface primer immobilized to the support, wherein individual library molecules further comprise a first left unique identification sequence (180) and/or a first right unique identification sequence (190);

b) providing a plurality of single-stranded splint strands (200), wherein individual single-stranded splint strands (200) in the plurality comprise, in a 5' to 3' order: (i) a first region (210) that hybridizes with the first left universal adaptor sequence (120) of individual library molecules, and (ii) a second region (220) that hybridizes with the first right universal adaptor sequence (130) of individual library molecules;

c) hybridizing the plurality of single-stranded splint strands (200) with the plurality of single-stranded nucleic acid library molecules (100), wherein the first region of the individual single-stranded splint strands (210) anneals to the first left universal adaptor sequences (120) of individual library molecules and the second region of the individual single-stranded splint strands (220) anneals to the first right universal adaptor sequence (130) of individual library molecules, thereby circularizing individual single-stranded nucleic acid library molecules and forming a plurality of library-splint complexes (300) having a nick between the terminal 5' and 3' ends of individual library molecules; and

d) ligating the nick in the plurality of library-splint complexes (300), thereby generating a plurality of covalently closed circular library molecules (400).

6. The method of claim 5, further comprising: (e) distributing the plurality of covalently closed circular library molecules (400) onto the support, wherein the support has a plurality of surface primers immobilized on the support, and hybridizing individual covalently closed circular library molecules (400) to individual immobilized surface primers, thereby immobilizing the plurality of covalently closed circular library molecules (400) to the support.

7. The method of claim 6, further comprising: (f) contacting the plurality of covalently closed circular library molecules (400) immobilized to the support at (e) with a plurality of strand-displacing polymerases and a plurality of nucleotides and conducting a rolling circle amplification reaction on the support using the plurality of surface primers as immobilized amplification primers and the plurality of covalently closed circular library molecules (400) as template molecules, thereby generating a plurality of immobilized nucleic acid concatemer molecules.

8. The method of claim 7, further comprising: sequencing the plurality of immobilized nucleic acid concatemer molecules.
9. The method of claim 8, wherein the sequencing comprises:
- a) contacting the plurality of immobilized nucleic acid concatemer molecules with (i) a plurality of sequencing polymerases and (ii) a plurality of soluble sequencing primers, thereby forming a plurality of complexed polymerases comprising a sequencing polymerase bound to a nucleic acid duplex, wherein the nucleic acid duplex comprises a nucleic acid concatemer molecule hybridized to a soluble sequencing primer;
 - b) contacting the plurality of complexed polymerases with a plurality of nucleotides and binding at least one nucleotide to a complexed polymerase, wherein the plurality of nucleotides comprises at least one nucleotide analog labeled with a fluorophore and having a removable chain terminating moiety at a sugar 3' position;
 - c) incorporating the at least one nucleotide into the 3' end of the hybridized soluble sequencing primers, thereby generating a plurality of nascent extended sequencing primers; and
 - d) detecting the incorporated nucleotide and identifying the nucleobase of the incorporated nucleotide.
10. The method of claim 8, wherein the sequencing comprises:
- a) contacting the plurality of immobilized nucleic acid concatemer molecules with (i) a plurality of sequencing polymerases and (ii) a plurality of soluble sequencing primers, thereby forming a plurality of first complexed polymerases comprising a sequencing polymerase bound to a nucleic acid duplex, wherein the nucleic acid duplex comprises a nucleic acid concatemer molecule hybridized to a soluble sequencing primer;
 - b) contacting the plurality of first complexed polymerases with a plurality of detectably labeled multivalent molecules and binding complementary nucleotide units of the plurality of detectably labeled multivalent molecules to at least two first complexed polymerases, thereby forming a plurality of multivalent-complexed polymerases, wherein incorporation of the complementary nucleotide units into the soluble sequencing primers of the plurality of multivalent-complexed polymerases is inhibited, wherein individual detectably labeled multivalent molecules comprise a

core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit;

- c) detecting the plurality of multivalent-complexed polymerases; and
- d) identifying the nucleo-bases of complementary nucleotide units that are bound to the plurality of first complexed polymerases, thereby determining the sequences of the nucleic acid concatemer template molecules.

11. The method of claim 10, further comprising:

- e) dissociating the plurality of multivalent-complexed polymerases and removing the plurality of first complexed polymerases and bound detectably labeled multivalent molecules, and retaining the plurality of nucleic acid duplexes, thereby generating a plurality of retained nucleic acid duplexes;
- f) contacting the plurality of retained nucleic acid duplexes of step (e) with a plurality of second sequencing polymerases and binding the plurality of second sequencing polymerases to the plurality of retained nucleic acid duplexes, thereby forming a plurality of second complexed polymerases comprising a second sequencing polymerase bound to a nucleic acid duplex; and
- g) contacting the plurality of second complexed polymerases with a plurality of nucleotides and binding complementary nucleotides from the plurality of nucleotides to at least two second complexed polymerases of step (f), thereby forming a plurality of nucleotide-complexed polymerases and incorporating the complementary nucleotides into the soluble sequencing primers of the nucleic acid duplex.

12. The method of claim 11, further comprising:

- h) detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases; and
- i) identifying the nucleo-bases of the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

13. The method of any one of claims 5-12, wherein the single-stranded nucleic acid library molecule (100) further comprises any one or any combination of:

- (vi) a first left sample index sequence (160), and/or
- (vii) a first right sample index sequence (170).

14. The method of any one of claims 5-13, wherein the first left universal adaptor sequence (120) and/or the second left universal adaptor sequence (140) comprises:

- (i) a universal binding sequence for a forward sequencing primer;
- (ii) a universal binding sequence for a reverse sequencing primer;
- (iii) a universal binding sequence for a first surface primer;
- (iv) a universal binding sequence for a second surface primer;
- (v) a universal binding sequence for a forward amplification primer;
- (vi) a universal binding sequence for a reverse amplification primer; and/or
- (vii) a universal binding sequence for a compaction oligonucleotide.

15. The method of any one of claims 5-14, wherein the first right universal adaptor sequence (130) and/or the second right universal adaptor sequence (150) comprises:

- (i) a universal binding sequence for a forward sequencing primer;
- (ii) a universal binding sequence for a reverse sequencing primer;
- (iii) a universal binding sequence for a first surface primer;
- (iv) a universal binding sequence for a second surface primer;
- (v) a universal binding sequence for a forward amplification primer;
- (vi) a universal binding sequence for a reverse amplification primer; and/or
- (vii) a universal binding sequence for a compaction oligonucleotide.

16. The method of any one of claims 5-15, wherein the first left index sequence (160) and/or the first right index sequence (170) are joined to a 3-mer random sequence at the 5' end, and wherein individual immobilized covalently closed circular library molecules comprise different 3-mer random sequences.

17. A method for generating a plurality of library-splint complexes (300) comprising:

- a) providing a plurality of single-stranded nucleic acid library molecules (100), wherein individual library molecules in the plurality comprise regions arranged in a 5' to 3' order: (i) a first left universal adaptor sequence (120) having a binding sequence for a first surface primer immobilized to a support; (ii) a second left universal adaptor sequence (140) having a binding sequence for a first sequencing

primer; (iii) a sequence of interest (110); (iv) a second right universal adaptor sequence (150) having a binding sequence for a second sequencing primer; and (v) a first right universal adaptor sequence (130) having a binding sequence for a second surface primer immobilized to the support, wherein the plurality of single-stranded nucleic acid library molecules (100) further comprise a first left unique identification sequence (180) and/or a first right unique identification sequence (190);

b) providing a plurality of single-stranded splint strands (200), wherein individual single-stranded splint strands (200) in the plurality comprise regions arranged in a 5' to 3' order: (i) a first region (210) having a universal binding sequence that hybridizes with the first left universal adaptor sequence (120) of a single-stranded nucleic acid library molecule (100), and (ii) a second region (220) having a universal binding sequence that hybridizes with the first right universal adaptor sequence (130) of the single-stranded nucleic acid library molecule (100);

c) hybridizing the plurality of single-stranded splint strands (200) with the plurality of single-stranded nucleic acid library molecules (100) under a condition suitable to hybridize the first region (210) of the single-stranded splint strand (200) to the first left universal adaptor sequence (120) of the linear single-stranded library molecule (100), and suitable to hybridize the second region (220) of the single-stranded splint strand (200) to first right universal adaptor sequence (130) of the linear single-stranded library molecule (100), thereby circularizing individual single-stranded nucleic acid library molecules to form a plurality of library-splint complexes (300) having a nick between the terminal 5' and 3' ends of a library molecule, wherein the nick is enzymatically ligatable.

18. The method of claim 17:

(i) wherein the plurality of single-stranded nucleic acid library molecules (100) further comprises a first left index sequence (160) and/or a first right index sequence (170); or

(ii) further comprising: d) ligating the nicks in the individual library-splint complexes (300), thereby generating a plurality of covalently closed circular library molecules (400).

19. The method of claim 18, further comprising: e) contacting the plurality of covalently closed circular library molecules (400) with at least one exonuclease

enzyme to remove the plurality of single-stranded splint strands (200) and retaining the plurality of covalently closed circular library molecules (400).

20. The method of claim 19, wherein the method further comprises: f) distributing the plurality of covalently closed circular library molecules (400) from step (e) onto the support, wherein the support comprises a plurality of second surface primers immobilized on the support, and hybridizing individual covalently closed circular library molecules (400) to individual second surface primers, thereby immobilizing the plurality of covalently closed circular library molecules (400) to the support.

21. The method of claim 20, further comprising: g) contacting the plurality of covalently closed circular library molecules (400) immobilized on the support with a plurality of strand-displacing polymerases and a plurality of nucleotides and conducting a rolling circle amplification reaction on the support using the plurality of second surface primers as immobilized amplification primers and the plurality of covalently closed circular library molecules (400) as template molecules, thereby generating a plurality of immobilized nucleic acid concatemer molecules.

22. The method of claim 20, wherein:

(i) the plurality of second surface primers on the support are located at pre-determined or random locations on the support, (ii) the plurality of second surface primers on the support are in fluid communication with each to permit flowing a solution of reagents onto the support so that the plurality of immobilized second surface primers can be simultaneously reacted with the solution of reagents in a massively parallel manner;

(iii) wherein the support comprises a glass or a plastic substrate; or

(iv) wherein the support is passivated with at least one hydrophilic polymer coating having a water contact angle of no more than 45 degrees.

23. The method of claim 22, wherein the at least one hydrophilic polymer coating 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)

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

24. The method of claim 22, wherein the solution of reagents comprises enzymes, nucleotides or divalent cations.

25. The method of claim 24:

(A) wherein the nucleotides comprise dATP, dGTP, dCTP, dTTP and/or dUTP; or

(B) wherein the plurality of immobilized nucleic acid concatemer molecules on the support are at a density of $10^4 - 10^8$ per mm^2 .

26. The method of any one of claims 21-25, further comprising: sequencing the plurality of immobilized nucleic acid concatemer molecules, wherein the sequencing comprises:

a) contacting the plurality of immobilized nucleic acid concatemer molecules with (i) a plurality of sequencing polymerases and (ii) a plurality of soluble sequencing primers and forming a plurality of complexed polymerases comprising a sequencing polymerase bound to a nucleic acid duplex, wherein the nucleic acid duplex comprises a concatemer molecule hybridized to a soluble sequencing primer;

b) contacting the plurality of complexed sequencing polymerases with a plurality of nucleotides and binding at least one nucleotide to a complexed polymerase, wherein the plurality of nucleotides comprises at least one nucleotide analog labeled with a fluorophore and having a removable chain terminating moiety at a sugar 3' position;

c) incorporating at least one nucleotide into the 3' end of the soluble sequencing primers, thereby generating a plurality of nascent extended sequencing primers; and

d) detecting the incorporated nucleotide and identifying the nucleobase of the incorporated nucleotide.

27. The method of claim 26, wherein the plurality of nucleotides:

(i) comprises a removable chain terminating moiety at the 3' sugar group, wherein the removable chain terminating moiety comprises an alkyl group, an alkenyl

group, an alkynyl group, an allyl group, an aryl group, a benzyl group, an azide group, an azido group, an O-azidomethyl group, an amine group, an amide group, a keto group, an isocyanate group, a phosphate group, a thio group, a disulfide group, a carbonate group, a urea group, or a 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;

(ii) comprises one type of nucleotide selected from the group consisting of dATP, dGTP, dCTP, dTTP and dUTP; or

(iii) comprises a mixture of any combination of two or more types of nucleotides selected from the group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

28. The method of any one of claims 21-25, further comprising: sequencing the plurality of immobilized nucleic acid concatemer molecules, wherein the sequencing comprises:

a) contacting the plurality of immobilized nucleic acid concatemer molecules with (i) a plurality of sequencing polymerases and (ii) a plurality of soluble sequencing primers and forming a plurality of first complexed polymerases comprising a sequencing polymerase bound to a nucleic acid duplex, wherein the nucleic acid duplex comprises a concatemer molecule hybridized to a soluble sequencing primer;

b) contacting the plurality of complexed polymerases with a plurality of detectably labeled multivalent molecules and binding complementary nucleotide units of the plurality of detectably labeled multivalent molecules to at least two of the first complexed polymerases, thereby forming a plurality of multivalent-complexed polymerases, wherein incorporation of the complementary nucleotide units into individual soluble sequencing primers is inhibited, wherein individual multivalent molecules comprise a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit;

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

d) identifying the nucleo-base of the complementary nucleotide units that are bound to the plurality of first complexed polymerases in the plurality of multivalent-complexed polymerases, thereby determining the sequences of the nucleic acid concatemer template molecules.

29. The method of claim 28, further comprising:

- e) dissociating the plurality of multivalent-complexed polymerases and removing the plurality of sequencing polymerases and the plurality of detectably labeled multivalent molecules, and retaining the plurality of nucleic acid duplexes;
- f) contacting the plurality of the nucleic acid duplexes of step (e) with a plurality of second sequencing polymerases and binding the plurality of second sequencing polymerases to the plurality of nucleic acid duplexes, thereby forming a plurality of second complexed polymerases comprising a second sequencing polymerase bound to a nucleic acid duplex; and
- g) contacting the plurality of second complexed polymerases with a plurality of nucleotides comprising at least one nucleotide analog having a removable chain terminating moiety at the sugar 3' position and 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, wherein the complementary nucleotides are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

30. The method of claim 29, further comprising:

- 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.

31. The method of any one of claims 28-30, wherein:

- (i) the plurality of nucleotides comprises a removable chain terminating moiety at the 3' sugar group, wherein the removable chain terminating moiety comprises an alkyl group, an alkenyl group, an alkynyl group, an allyl group, an aryl group, a benzyl group, an azide group, an azido group, an O-azidomethyl group, an amine group, an amide group, a keto group, an isocyanate group, a phosphate group, a thio group, a disulfide group, a carbonate group, a urea group, or a 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;

(ii) the plurality of nucleotides comprise one type of nucleotide selected from the group consisting of dATP, dGTP, dCTP, dTTP and dUTP; or

(iii) the plurality of nucleotides comprises a mixture of any combination of two or more types of nucleotides selected from the group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

32. The method of any one of claims 28-31, wherein:

(i) the multiple nucleotide arms attached to the core of individual multivalent molecules have 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

(ii) the plurality of multivalent molecules comprise a mixture of any combination of two or more types of multivalent molecules having nucleotide units selected from the group consisting of dATP, dGTP, dCTP, dTTP and dUTP.