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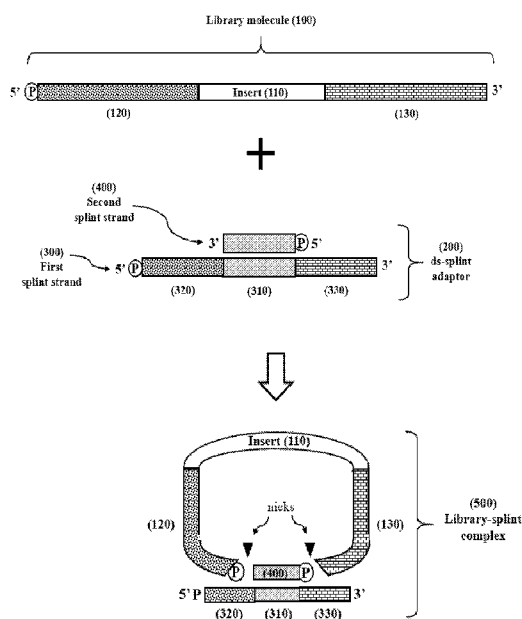


FIG. 1

(57) Abstract: The present disclosure provides compositions compris-  
ing nucleic acid double-stranded splint adaptors, including kits, and  
methods that employ the double-stranded splint adaptors. The dou-  
ble-stranded splint adaptors (200) can be used in a one-pot, multi-en-  
zyme reaction to introduce one or more new adaptor sequences in-  
to a library molecule. The double-stranded splint adaptor (200) com-  
prises a first splint strand (long splint strand (300)) and a second  
splint strand (short splint strand (400)), where the first and second  
splint strands are hybridized together to form the double-stranded splint  
adaptor (200) having a double-stranded region and two flanking sin-  
gle-stranded regions. The second splint strand (400) carries the new  
adaptor sequence(s) to be introduced, such as for example a universal  
binding sequence and/or an index sequence.

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## **DOUBLE-STRANDED SPLINT ADAPTORS AND METHODS OF USE**

### **RELATED APPLICATIONS**

**[0001]** This application claims the priority to and claims the benefit of U.S. Provisional Patent Application No. 63/316,784, filed March 4, 2022 and U.S. Patent Application No. 17/725,065, filed April 20, 2022, the contents of each of which are incorporated by reference herein in their entireties.

### **INCORPORATION BY REFERENCE OF SEQUENCE LISTING**

**[0002]** The contents of the electronic sequence listing (ELEM-003\_001WO\_SequenceListing\_ST26.xml; Size: 65,027 bytes; and Date of Creation: March 3, 2023) are herein incorporated by reference in its entirety.

### **TECHNICAL FIELD**

**[0003]** The present disclosure provides compositions comprising nucleic acid double-stranded splint adaptors, and methods for using the double-stranded splint adaptors. The double-stranded splint adaptors can hybridize to portions of library molecules to form library-splint complexes having nicks, where the nicks can be ligated to form covalently closed circular molecules which can be subjected to downstream amplification and sequencing workflows.

### **BACKGROUND**

**[0004]** The present disclosure relates to, preparing and sequencing polynucleotides from libraries of covalently closed circular molecules. Improvements in next generation sequencing technology have greatly increased sequencing speed and data output, resulting in the high sample throughput of current sequencing platforms. Efficient preparation of closed circular library molecules having target sequences is important for downstream amplification and sequencing workflows. Another aspect of increasing sequencing throughput is the addition of unique index sequences to DNA fragments during library preparation, which allows large number of libraries to be pooled and sequenced simultaneously during each sequencing run. Accordingly, there is a need for alternative methods for producing and sequencing a circular library molecules containing target sequences and unique index

sequences, which are compatible for downstream next generation sequencing technologies. Provided herein are compositions, methods and kits addressing this need.

## SUMMARY

**[0005]** The disclosure provides a library-splint complex (500) 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 double-stranded splint adaptor (200) which includes a first splint strand (long splint strand (300)) and a second splint strand (short splint strand (400)), wherein the double-stranded splint adaptor (200) includes a double-stranded region and two flanking single-stranded regions, wherein the first splint strand comprises a first region (320), an internal region (310), and a second region (330); wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the first region of the first splint strand (320) is hybridized to the at least first left universal adaptor sequence (120) of the library molecule, and wherein a second region of the first splint strand (330) is hybridized to the at least first right universal sequence (130) of the library molecule, thereby circularizing the library molecule to generate a library-splint complex (500).

**[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 index sequence (160). In some embodiments, the nucleic acid library molecule (100) further comprises: a first right 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: (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.

**[0009]** In some embodiments, 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 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.

**[0010]** In some embodiments, the second splint strand (400) includes at least two sub-regions, the first sub-region comprising a universal binding sequence for a third surface primer, and the second sub-region comprising a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize to (or at least exhibit very little hybridization to) the first and second surface primers.

**[0011]** In some embodiments, the second splint strand (400) further comprises an optional third sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN).

**[0012]** In some embodiments, the first splint strand (300) includes an internal region (310) comprising at least two sub-regions, the fourth sub-region comprising a universal binding sequence for a third surface primer and the fourth sub-region hybridizes to the first sub-region of the second splint strand (400), and the fifth sub-region comprising a universal binding sequence for a fourth surface primer and the fifth sub-region hybridizes to the second sub-region of the second splint strand (400), wherein the fourth and fifth sub-regions do not hybridize to (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the first splint strand (300) includes an internal region (310) which further comprises a sixth sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN), wherein the sixth sub-region hybridizes to the third sub-region of the second splint strand (400).

**[0013]** The disclosure provides a library-splint complex (500) comprising: **a)** 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 (120); (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 (130); **b)** a first splint strand (300) comprising components arranged in a 5' to 3' order: a first region (320); an internal region (310); and a second region (330); and **c)** a second splint strand (400) comprising sub-regions arranged in a 3' to 5' order: a first sub-region having a universal binding sequence for a third surface primer; and a second sub-region having a universal binding sequence for a fourth surface primer; wherein the first splint strand (300) is hybridized to portions of the library molecule (100) thereby circularizing the library molecule to generate a library-splint complex (500), such that the first region (320) of the first splint strand is hybridized to the binding sequence for the first surface primer (120), and the third region (330) of the first splint strand is hybridized to the binding sequence for the second surface primer (130), wherein the second splint strand (400) is hybridized to the internal region (310) of the first splint strand (300), wherein the library-splint complex (500) comprises a first nick between the 5' end of the library molecule and the 3' end of the second splint strand, wherein the library-splint complex (500) comprises a second nick between the 5' end of the second splint strand and the 3' end of the library molecule, and wherein the first and second nicks are enzymatically ligatable.

**[0014]** In some embodiments, the library-splint complex (500) further comprising a plurality of library-splint complexes (500), wherein the sequence of interest (110) of individual library-splint complexes in the plurality comprise the same sequence of interest or different sequences of interest.

**[0015]** The disclosure provides a reaction mixture comprising the plurality of library-splint complexes (500) of the disclosure and T4 polynucleotide kinase. The disclosure provides a reaction mixture comprising the plurality of library-splint complexes (500) of the disclosure and a ligase enzyme. The disclosure provides a reaction mixture comprising the plurality of library-splint complexes (500) of the disclosure, T4 polynucleotide kinase, and a ligase enzyme. In some embodiments, the ligase enzyme comprises T7 DNA ligase, T3 ligase, T4 ligase, or Taq ligase.

**[0016]** The disclosure provides a covalently closed circular library molecule (600) comprising: a sequence of interest (110), at least a first left universal adaptor sequence (120),

at least a first right universal adaptor sequence (130), and a second splint strand sequence (400).

**[0017]** In some embodiments, the covalently closed circular library molecule (600) of the disclosure further comprises: a second left universal adaptor sequence (140). In some embodiments, the covalently closed circular library molecule (600) of the disclosure further comprises: a second right universal adaptor sequence (150). In some embodiments, the covalently closed circular library molecule (600) of the disclosure further comprises: a first left index sequence (160). In some embodiments, the covalently closed circular library molecule (600) of the disclosure further comprises: a first right index sequence (170). In some embodiments, the covalently closed circular library molecule (600) of the disclosure further comprises: a first left unique identification sequence (180). In some embodiments, the covalently closed circular library molecule (600) of the disclosure further comprises: a first right unique identification sequence (190).

**[0018]** In some embodiments, the covalently closed circular library molecule (600) of the disclosure further comprises: any one or any combination of two or more: (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).

**[0019]** 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.

**[0020]** In some embodiments, 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 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.

**[0021]** In some embodiments, the closed circular library molecule (600) of the disclosure is hybridized to a first splint strand (long splint strand (300)) which comprises a first region

(320), an internal region (310), and a second region (330), wherein the second splint strand sequence (400) of the covalently closed circular library molecule is hybridized to the internal region (310) of the first splint strand, wherein the first region of the first splint strand (320) is hybridized to the at least first left universal adaptor sequence (120) of the library molecule, and wherein a second region of the first splint strand (330) is hybridized to the at least first right universal sequence (130) of the library molecule.

**[0022]** In some embodiments, the second splint strand (400) includes at least two sub-regions, the first sub-region comprising a universal binding sequence for a third surface primer, and the second sub-region comprising a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize to (or at least exhibit very little hybridization to) the first and second surface primers.

**[0023]** In some embodiments, the second splint strand (400) further comprises an optional third sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN).

**[0024]** In some embodiments, the first splint strand (300) includes an internal region (310) comprises at least two sub-regions, the fourth sub-region comprising a universal binding sequence for a third surface primer and the fourth sub-region hybridizes to the first sub-region of the second splint strand (400), and the fifth sub-region comprising a universal binding sequence for a fourth surface primer and the fifth sub-region hybridizes to the second sub-region of the second splint strand (400), wherein the fourth and fifth sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the first splint strand (300) includes an internal region (310) which further comprises a sixth sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN), wherein the sixth sub-region hybridizes to the third sub-region of the second splint strand (400).

**[0025]** The disclosure provides a single-stranded covalently closed circular molecule (600) comprising: **(i)** a first left universal adaptor sequence (120) having a binding sequence for a first surface primer (120); **(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; **(v)** a first right universal adaptor sequence (130) having a binding sequence for a second surface primer (130); and **(vi)** a second splint strand (400), wherein the covalently closed circular molecule (600) is optionally hybridized to the first splint strand (300).

**[0026]** In some embodiments, the covalently closed circular library molecule (600) further comprises a plurality of covalently closed circular library molecules (600), wherein the sequence of interest (110) of individual covalently closed circular library molecules (600) in the plurality comprise the same sequence of interest or different sequences of interest.

**[0027]** The disclosure provides a reaction mixture comprising the plurality of plurality of covalently closed circular library molecules (600) of the disclosure, wherein individual covalently closed circular library molecules (600) are hybridized to a first splint strand (300). In some embodiments, plurality of covalently closed circular library molecules (600) and at least one exonuclease enzyme. In some embodiments, the exonuclease enzyme comprises exonuclease I, thermolabile exonuclease I, or T7 exonuclease.

**[0028]** The disclosure provides a kit, comprising: a double-stranded splint adaptor (200) which includes **(i)** a first splint strand (long splint strand (300)) and **(ii)** a second splint strand (short splint strand (400)), wherein the first splint strand (300) can hybridize to the second splint strand (400) to form a double-stranded region and two flanking single-stranded regions, wherein the first splint strand (300) comprises a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) can hybridize to the second splint strand (400). In some embodiments, the first splint strand (300) and the second splint strand (400) are hybridized to each other to form a double-stranded splint adaptor (200) having a double-stranded region and two flanking single-stranded regions, and wherein the internal region of the first splint strand (310) can hybridize to the second splint strand (400).

**[0029]** In some embodiments, the kit of the disclosure is for the use in circularizing a single-stranded nucleic acid library molecule (100) having 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). In some embodiments, the first region of the first splint strand (320) can hybridize to at least first left universal adaptor sequence (120) of the library molecule (100), and wherein a second region of the first splint strand (330) can hybridize to the at least first right universal sequence (130) of the library molecule. In some embodiments, the at least first left universal adaptor sequence (120) of the library molecule (100) 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.

**[0030]** In some embodiments, the at least first right universal adaptor sequence (130) of the library molecule (100) 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.

**[0031]** In some embodiments, the second splint strand (400) includes at least two sub-regions, the first sub-region comprising a universal binding sequence for a third surface primer, and the second sub-region comprising a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the second splint strand (400) further comprises an optional third sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN).

**[0032]** In some embodiments, the first splint strand (300) includes an internal region (310) comprising at least two sub-regions, the fourth sub-region comprising a universal binding sequence for a third surface primer and the fourth sub-region hybridizes to the first sub-region of the second splint strand (400), and the fifth sub-region comprising a universal binding sequence for a fourth surface primer and the fifth sub-region hybridizes to the second sub-region of the second splint strand (400), wherein the fourth and fifth sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the first splint strand (300) includes an internal region (310) which further comprises a sixth sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN), wherein the sixth sub-region hybridizes to the third sub-region of the second splint strand (400).

**[0033]** In some embodiments, the kit of the disclosure further comprises a T4 polynucleotide kinase. In some embodiments, the kit of the disclosure further comprises a ligase enzyme. In some embodiments, the kit of the disclosure further comprises a T4 polynucleotide kinase and a ligase enzyme. In some embodiments, the ligase enzyme comprises T7 DNA ligase, T3 ligase, T4 ligase, or Taq ligase.

**[0034]** The disclosure provides a method for forming a plurality of library-splint complexes (500) comprising: **a)** providing a plurality of double-stranded splint adaptors wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the double-stranded splint adaptor includes a double-stranded region and two flanking single-stranded regions, wherein the first splint strand comprises a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400); and **b)** hybridizing the plurality of double-stranded splint adaptors with a plurality of single-stranded nucleic acid library molecules (100) wherein individual library molecules include 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), wherein the hybridizing is conducted under a condition suitable to hybridize the first region of the first splint strand (320) to the at least first left universal adaptor sequence (120) of the library molecule, and the condition is suitable to hybridize the second region of the first splint strand (330) to the at least first right universal adaptor sequence (130) of the library molecule, thereby circularizing the plurality of library molecules to form a plurality of library-splint complexes (500).

**[0035]** In some embodiments, the individual library-splint complexes (500) in the plurality comprise a first nick between the 5' end of the library molecule and the 3' end of the second splint strand, and wherein individual library-splint complexes (500) in the plurality comprise a second nick between the 5' end of the second splint strand and the 3' end of the library molecule. In some embodiments, the individual nucleic acid library molecules (100) in the plurality further comprise: a second left universal adaptor sequence (140). In some embodiments, the individual nucleic acid library molecules (100) in the plurality further comprise: a second right universal adaptor sequence (150). In some embodiments, the individual nucleic acid library molecules (100) in the plurality further comprise: a first left index sequence (160). In some embodiments, the individual nucleic acid library molecules (100) in the plurality further comprise: a first right index sequence (170). In some embodiments, the individual nucleic acid library molecules (100) in the plurality further comprise: a first left unique identification sequence (180). In some embodiments, the individual nucleic acid library molecules (100) in the plurality further comprise: a first right unique identification sequence (190).

**[0036]** In some embodiments, the individual nucleic acid library molecules (100) in the plurality further comprise 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). In some embodiments, the first and/or the second left universal adaptor sequence 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.

**[0037]** In some embodiments, the first and/or the second right universal adaptor sequence 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.

**[0038]** In some embodiments, the second splint strand (400) includes at least two sub-regions, the first sub-region comprising a universal binding sequence for a third surface primer, and the second sub-region comprising a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize to (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the second splint strand (400) further comprises an optional third sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN).

**[0039]** In some embodiments, the first splint strand (300) includes an internal region (310) comprising at least two sub-regions, the fourth sub-region comprising a universal binding sequence for a third surface primer and the fourth sub-region hybridizes to the first sub-region of the second splint strand (400), and the fifth sub-region comprising a universal binding sequence for a fourth surface primer and the fifth sub-region hybridizes to the second sub-region of the second splint strand (400), wherein the fourth and fifth sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the first splint strand (300) includes an internal region (310) which further comprises a sixth sub-region which comprises a sample index sequence having 5-20

bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN), wherein the sixth sub-region hybridizes to the third sub-region of the second splint strand (400).

**[0040]** The disclosure provides a method for forming a plurality of library-splint complexes (500) comprising: **a)** providing a plurality of double-stranded splint adaptors (200) wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the first splint strand (300) comprises regions arranged in a 5' to 3' order a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the second splint strand comprises regions arranged in a 5' to 3' order **(i)** a second sub-region having a universal binding sequence for a fourth surface primer, and **(ii)** a first sub-region having a universal binding sequence for a third surface primer; and **b)** hybridizing the plurality of double-stranded splint adaptors with a plurality of single-stranded nucleic acid library molecules (100) wherein individual library molecules 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 (120); **(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 (130), wherein the hybridizing is conducted under a condition suitable to hybridize portions of the first splint strand (300) to portions of the library molecule (100) thereby circularizing the library molecule to generate a library-splint complex (500), such that the first region (320) of the first splint strand is hybridized to the binding sequence for the first surface primer (120), and the third region (330) of the first splint strand is hybridized to the binding sequence for the second surface primer (130), wherein the library-splint complex (500) comprises a first nick between the 5' end of the library molecule and the 3' end of the second splint strand (300), wherein the library-splint complex (500) comprises a second nick between the 5' end of the second splint strand (300) and the 3' end of the library molecule (100), and wherein the first and second nicks are enzymatically ligatable.

**[0041]** In some embodiments, 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). In some embodiments, the plurality of single-stranded nucleic acid library molecules (100) further comprises a first left unique identification sequence (180) and/or a first right unique identification sequence (190).

**[0042]** In some embodiments, the method further comprises: contacting the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a T4 polynucleotide kinase enzyme under a condition suitable to phosphorylate the 5' ends of the plurality of double-stranded splint adaptors (200) and/or the plurality of single-stranded nucleic acid library molecules (100).

**[0043]** In some embodiments, the method further comprises: contacting the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a ligase, under a condition suitable to enzymatically ligate the first and second nicks, thereby generating a plurality of covalently closed circular library molecules (600) each hybridized to the first splint strand (300).

**[0044]** In some embodiments, the method further comprising conducting the sequential steps of: **a)** contacting the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a T4 polynucleotide kinase enzyme under a condition suitable to phosphorylate the 5' ends of the plurality of double-stranded splint adaptors (200) and the plurality of single-stranded nucleic acid library molecules (100); and **b)** contacting the phosphorylated double-stranded splint adaptors (200) and the phosphorylated single-stranded nucleic acid library molecules (100) with a ligase under a condition suitable to enzymatically ligate the first and second nicks, thereby generating a plurality of covalently closed circular library molecules (600) each hybridized to the first splint strand (300).

**[0045]** In some embodiments, the method further comprises conducting the essentially simultaneous steps of contacting the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a (i) T4 polynucleotide kinase enzyme and (ii) a ligase enzyme, under a condition suitable to phosphorylate the 5' ends of the plurality of double-stranded splint adaptors (200) and the plurality of single-stranded nucleic acid library molecules (100), and the conditions are suitable to enzymatically ligate the first and second nicks, thereby generating a plurality of covalently closed circular library molecules (600) each hybridized to the first splint strand (300).

**[0046]** In some embodiments, the method further comprises contacting the plurality of covalently closed circular library molecules (600) with at least one exonuclease enzyme to remove the plurality of first splint strands (300) and retaining the plurality of covalently closed circular library molecules (600). In some embodiments, the at least one exonuclease enzyme comprises exonuclease I, thermolabile exonuclease I and/or T7 exonuclease.

**[0047]** In some embodiments, the individual covalently closed circular library molecules (600) in the plurality comprise a second splint strand region (400) which includes a universal binding sequence for a third surface primer, and wherein the method further comprises: distributing the retained plurality of covalently closed circular library molecules (600) onto a support having a plurality of the third surface primers immobilized on the support, under a condition suitable for hybridizing individual covalently closed circular library molecules (600) to individual immobilized third surface primers thereby immobilizing the plurality of covalently closed circular library molecules (600).

**[0048]** In some embodiments, the method further comprises: contacting the plurality of immobilized covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of third surface primers as immobilized amplification primers and the plurality of covalently closed circular library molecules (600) as template molecules, thereby generating a plurality of immobilized nucleic acid concatemer molecules. In some embodiments, the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0049]** In some embodiments, the first (120) or the second (140) left universal adaptor sequence of individual library molecules in the plurality comprise a universal binding sequence for a forward amplification primer, and the method further comprises: **a)** hybridizing a plurality of soluble forward amplification primers to the first or second left universal adaptor sequence which comprise the universal binding sequence for a forward amplification primer; and **b)** conducting a rolling circle amplification reaction thereby generating a plurality of nucleic acid concatemer molecules.

**[0050]** In some embodiments, the first (130) or the second (150) right universal adaptor sequence of individual library molecules in the plurality comprise a universal binding sequence for a forward amplification primer, and the method further comprises: **a)** hybridizing a plurality of soluble forward amplification primers to the first or second right universal adaptor sequence which comprise the universal binding sequence for a forward amplification primer; and **b)** conducting a rolling circle amplification reaction thereby generating a plurality of nucleic acid concatemer molecules each hybridized to a covalently closed circular library molecule (600).

**[0051]** In some embodiments, the individual concatemer molecules in the plurality comprise a second splint strand region (400) having a universal binding sequence for third surface primer, wherein individual concatemer molecules are hybridized to a covalently

closed circular library molecule (600), and the method further comprises: distributing the plurality of concatemer molecules wherein onto a support having a plurality of the third surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized third surface primers thereby immobilizing the plurality of concatemer molecules.

**[0052]** In some embodiments, the method further comprises: contacting the immobilized plurality of concatemer molecules (which are hybridized to a covalently closed circular library molecule (600)) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules. In some embodiments, the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0053]** In some embodiments, individual covalently closed circular library molecules (600) in the plurality are hybridized to a first splint strand (300), and the method further comprises: contacting the plurality of covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides under a condition suitable for conducting a rolling circle amplification reaction using the first splint strand (300) as an amplification primer thereby generating a plurality of concatemer molecules.

**[0054]** In some embodiments, individual concatemer molecules in the plurality comprise a second splint strand region (400) which includes a universal binding sequence for third surface primer, and the method further comprises: distributing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600) onto a support having a plurality of the third surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized third surface primers thereby immobilizing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600).

**[0055]** In some embodiments, the method further comprises: contacting the plurality of immobilized concatemer molecules with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules. In some embodiments, the plurality of nucleotides comprises dATP,

dGTP, dCTP, dTTP and/or dUTP. In some embodiments, the support further comprises a plurality of the fourth surface primers immobilized on the support.

**[0056]** In some embodiments, the plurality of immobilized nucleic acid concatemer molecules comprise at least two second splint strand regions (400) each having a universal binding sequence for the third and fourth surface primer, and the method further comprises: incubating the plurality of immobilized nucleic acid concatemer molecules under a condition suitable for hybridizing at least one second splint strand region (400) of at least one concatemer molecule to an immobilized fourth surface primer thereby pinning down at least one portion of the immobilized concatemer molecule to the support.

**[0057]** In some embodiments, the method further comprises: sequencing the plurality of immobilized concatemers, the method comprising: **a)** contacting the plurality of immobilized concatemer molecules with (i) a plurality of sequencing polymerases and (ii) a plurality of the soluble 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 concatemer molecule hybridized to a soluble 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 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.

**[0058]** In some embodiments, the method further comprises: sequencing the plurality of immobilized concatemers, the method comprising: **a)** contacting the plurality of immobilized concatemer molecules with (i) a plurality of sequencing polymerases and (ii) a plurality of the soluble sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each 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 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.

**[0059]** In some embodiments, the method further comprises: **e)** dissociating the plurality of multivalent-complexed polymerases and removing the plurality of first sequencing polymerases and their bound multivalent molecules, and retaining the plurality of nucleic acid duplexes; **f)** contacting the plurality of the retained nucleic acid duplexes of step **(e)** with a plurality of second sequencing polymerases, wherein the contacting is conducted under a condition suitable for binding the plurality of second sequencing polymerases to the plurality of the retained nucleic acid duplexes, thereby forming a plurality of second complexed polymerases each comprising a second sequencing polymerase bound to a retained nucleic acid duplex; **g)** contacting the plurality of second complexed polymerases with a plurality of nucleotides, wherein the contacting is conducted under a condition suitable for binding complementary nucleotides from the plurality of nucleotides to at least two of the second complexed polymerases of step **(f)** thereby forming a plurality of nucleotide-complexed polymerases and the condition is suitable for promoting incorporation of the bound complementary nucleotides into the sequencing primers of the nucleotide-complexed polymerases. In some embodiments, the plurality of nucleotides includes at least one nucleotide analog labeled with a fluorophore and having a removable chain terminating moiety at the sugar 3' position.

**[0060]** In some embodiments, the method further comprises: **h)** detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases. In some embodiments, the method further comprises: **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.

**[0061]** The disclosure provides a method for forming at least one avidity complex, comprising: **a)** binding a first universal nucleic acid primer, a first DNA polymerase, and a

first multivalent molecule to a first portion of any of the concatemer molecules of any one of claims 80, 85, 89 or 92, thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first DNA polymerase; and **b)** binding a second universal nucleic acid primer, a second DNA polymerase, and the first multivalent molecule to a second portion of the same concatemer template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the first multivalent molecule binds to the second DNA polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms an avidity complex, wherein the first multivalent molecule comprises a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit, and wherein the concatemer molecule comprises two or more tandem repeat sequences of a sequence of interest (110) and a universal primer binding site that binds the first and second universal nucleic acid primers.

**[0062]** The disclosure provides a method for sequencing by forming at least one avidity complex, comprising: **a)** binding a first universal nucleic acid primer, a first DNA polymerase, and a first multivalent molecule to a first portion of any of the concatemer molecules of the disclosure, thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first DNA polymerase; **b)** binding a second universal nucleic acid primer, a second DNA polymerase, and the first multivalent molecule to a second portion of the same concatemer template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the first multivalent molecule binds to the second DNA polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms an avidity complex, wherein the first multivalent molecule comprises a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit, wherein the concatemer molecule comprises two or more tandem repeat sequences of a sequence of interest (110) and a universal primer binding site that binds the first and second universal nucleic acid primers, and 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; **c)** detecting the first and second binding complexes on the same concatemer template molecule, and **d)** identifying the first nucleotide unit in the first binding complex thereby determining the sequence of the first portion of the concatemer template molecule, and identifying the second nucleotide unit in the second binding complex thereby determining the sequence of the second portion of the concatemer template molecule.

**[0063]** The disclosure provides a method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources, comprising: **a)** providing two or more populations of single-stranded nucleic acid library molecules (100), each population of library molecules (100) contained in a separate compartment, wherein the nucleic acid library molecules in a given population comprise **(i)** a first left universal adaptor sequence (120) having a binding sequence for a first surface primer, **(ii)** a second left universal adaptor sequence (140) having a binding sequence for a first sequencing primer; **(iii)** a sequence of interest (110) isolated from a sample source, **(iv)** a second right universal adaptor sequence (150) having a binding sequence for a second sequencing primer; **(v)** a first right universal adaptor sequence (130) having a binding sequence for a second surface primer, and **(vii)** a first left index sequence (160) and/or a first right index sequence (170), wherein the sequences of the left (160) and/or right (170) indexes separately or in combination identify the sample source from which the sequences of interest (110) have been isolated; **b)** providing a plurality of double-stranded splint adaptors (200) wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the first splint strand (300) comprises regions arranged in a 5' to 3' order a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the second splint strand comprises regions arranged in a 5' to 3' order **(i)** a second sub-region having a universal binding sequence for a fourth surface primer, and **(ii)** a first sub-region having a universal binding sequence for a third surface primer; **c)** contacting in the separate compartments the population of single-stranded nucleic acid library molecules (100) with a plurality of double-stranded splint adaptors (200), wherein the contacting is conducted under a condition suitable to hybridize portions of the first splint strand (300) to portions of the library molecules (100) thereby circularizing the library molecules to generate a population of library-splint complexes (500), such that the first region (320) of an individual first splint strand is hybridized to the binding sequence for the first surface primer (120) of an individual library molecule (100), and the third region (330) of the individual first splint strand is hybridized to the binding sequence for the second surface primer (130) of the individual library molecule (100), wherein each of the library-splint complexes (500) comprise a first nick between the 5' end of the library molecule and the 3' end of the second splint strand (300), wherein each of the library-splint complexes (500) comprises a second nick between the 5' end of the second splint strand (300) and the 3' end of the library molecule (100), and wherein the first and second nicks are enzymatically ligatable; **d)**

contacting in the separate compartments the populations of library-splint complexes (500) with a ligase, under a condition suitable to enzymatically ligate the first and second nicks, thereby generating two or more populations of covalently closed circular library molecules (600) each hybridized to the first splint strand (300); and **e)** pooling together the two or more populations of covalently closed circular library molecules (600) from the separate compartments to generate a multiplex mixture of covalently closed circular library molecules (600) which comprise the multiplex mixture of sequences-of-interest isolated from a plurality of sample sources.

**[0064]** In some embodiments, the individual covalently closed circular library molecules (600) in the multiplex mixture of step (e) comprise a second splint strand region (400) which includes a universal binding sequence for a third surface primer, and the method further comprises: **a)** distributing the multiplex mixture of covalently closed circular library molecules (600) onto a support having a plurality of the third surface primers immobilized on the support, under a condition suitable for hybridizing individual covalently closed circular library molecules (600) to individual immobilized third surface primers thereby immobilizing the plurality of covalently closed circular library molecules (600); and **b)** contacting the multiplex mixture of immobilized covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of third surface primers as immobilized amplification primers and the plurality of covalently closed circular library molecules (600) as template molecules, thereby generating a plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0065]** In some embodiments, the method further comprises: conducting a rolling circle amplification reaction by: **a)** contacting the two or more populations of covalently closed circular library molecules (600) that are hybridized to the first splint strand (300) of step (d) or (e) with at least one exonuclease enzyme under a condition suitable to remove the plurality of first splint strands (300) and retaining the population of covalently closed circular library molecules (600); and **b)** contacting the retained two or more populations of covalently closed circular library molecules (600) with a plurality of soluble amplification primers, a plurality of strand-displacing polymerases, and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction thereby generating a plurality of nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0066]** In some embodiments, the method further comprises: **a)** distributing the plurality of concatemer molecules onto a support having a plurality of the third surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized third surface primers thereby immobilizing the plurality of concatemer molecules; and **b)** contacting the immobilized plurality of concatemer molecules (which are hybridized to a covalently closed circular library molecule (600)) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0067]** In some embodiments, the method further comprises: conducting a rolling circle amplification reaction by contacting the two or more populations of covalently closed circular library molecules (600) each hybridized to the first splint strand (300) of step **(d)** or **(e)** with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction thereby generating a plurality of concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0068]** In some embodiments, the method further comprises: **a)** distributing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600) onto a support having a plurality of the third surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized third surface primers thereby immobilizing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600); and **b)** contacting the plurality of immobilized concatemer molecules with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0069]** In some embodiments, the method further comprises: sequencing the insert region (110) and the first left index sequence (160) and/or the first right index sequence (170) of the plurality of immobilized nucleic acid concatemer molecules, wherein the sequences of the

insert region (110) along with the first left index sequence (160) and/or first right index sequence (170) can be used to identify the sample source of the sequence of interest.

**[0070]** In some embodiments, the sequencing reaction comprises: **a)** binding the plurality of immobilized nucleic acid concatemer molecules with a plurality of sequencing primers, a plurality of sequencing polymerases, and a plurality of detectably labeled nucleotide analogs each comprising a 2' or 3' chain terminating moiety, under a condition suitable for incorporating the detectably labeled nucleotide analogs into the 3' end of the sequencing primers; **b)** detecting the bound detectably labeled nucleotide analog; and **c)** identifying the nucleobase of the detectably labeled nucleotide analog. In some embodiments, the sequencing reaction employs a two-stage sequencing reaction, wherein the first stage comprises: **a)** binding the plurality of immobilized nucleic acid concatemer molecules with a plurality of sequencing primers, a plurality of a first sequencing polymerase, and a plurality of detectably labeled multivalent molecules, under a condition suitable to bind individual concatemer molecules to a sequencing primer, a first sequencing polymerase, and a detectably labeled multivalent molecule (e.g., forming a plurality of multivalent-complexed polymerases); **b)** detecting the bound detectably labeled multivalent molecule; and **c)** identifying the nucleobase of the detectably labeled multivalent molecule. In some embodiments, the binding of the detectably labeled multivalent molecules to the plurality of immobilized nucleic acid concatemer molecules forms at least one avidity complex.

**[0071]** In some embodiments, the method further comprises conducting a second stage which comprises: **d)** removing the plurality of first sequencing polymerases and the plurality of bound multivalent molecules from the plurality of immobilized nucleic acid concatemer molecules and retaining the immobilized concatemer molecules each hybridized to a sequencing primer (nucleic acid duplex); and **e)** contacting the retained immobilized concatemer molecules and hybridized sequencing primers with a plurality of second polymerases and a plurality of nucleotides under a condition suitable for incorporating the nucleotides into the 3' end of the sequencing primers.

**[0072]** The disclosure provides a method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources, comprising: **a)** providing two or more populations of single-stranded nucleic acid library molecules (100), each population of library molecules (100), each population of single-stranded nucleic acid library molecules is contained in a separate compartment, wherein the nucleic acid library molecules in a given population comprise **(i)** a first left universal adaptor sequence (120) having a binding sequence for a first surface primer, **(ii)** a second left universal adaptor sequence (140) having

a binding sequence for a first sequencing primer; **(iii)** a sequence of interest (110) isolated from a sample source, **(iv)** a second right universal adaptor sequence (150) having a binding sequence for a second sequencing primer; **(v)** a first right universal adaptor sequence (130) having a binding sequence for a second surface primer, and **(vi)** a first left index sequence (160) and/or a first right index sequence (170), wherein the sequences of the left (160) and/or right (170) indexes separately or in combination identify the sample source from which the sequences of interest (110) are isolated; **b)** pooling together the two or more populations of single-stranded nucleic acid library molecules (100) into the same compartment; **c)** adding to the compartment of step (b) a plurality of double-stranded splint adaptors (200) wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the first splint strand (300) comprises regions arranged in a 5' to 3' order a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the second splint strand comprises regions arranged in a 5' to 3' order of **(i)** a second sub-region having a universal binding sequence for a fourth surface primer, and **(ii)** a first sub-region having a universal binding sequence for a third surface primer; **d)** incubating the population of single-stranded nucleic acid library molecules (100) and the plurality of double-stranded splint adaptors (200) which are in the same compartment of step (c) under a condition suitable to hybridize portions of the first splint strand (300) to portions of the library molecules (100) thereby circularizing the library molecules to generate a population of library-splint complexes (500), such that the first region (320) of an individual first splint strand is hybridized to the binding sequence for the first surface primer (120) of an individual library molecule (100), and the third region (330) of the individual first splint strand is hybridized to the binding sequence for the second surface primer (130) of the individual library molecule (100), wherein each of the library-splint complexes (500) comprise a first nick between the 5' end of the library molecule and the 3' end of the second splint strand (300), wherein each of the library-splint complexes (500) comprises a second nick between the 5' end of the second splint strand (300) and the 3' end of the library molecule (100), and wherein the first and second nicks are enzymatically ligatable; and **e)** contacting the populations of library-splint complexes (500) in the compartment of step (d) with a ligase, under a condition suitable to enzymatically ligate the first and second nicks, thereby generating a population of covalently closed circular library molecules (600) each hybridized to the first splint strand (300), thereby generating a

multiplex mixture of covalently closed circular library molecules (600) which comprise the multiplex mixture of sequences-of-interest isolated from a plurality of sample sources.

**[0073]** In some embodiments, the individual covalently closed circular library molecules (600) in the multiplex mixture of step (e) comprise a second splint strand region (400) which includes a universal binding sequence for a third surface primer, and the method further comprises: **a)** distributing the plurality of covalently closed circular library molecules (600) onto a support having a plurality of the third surface primers immobilized on the support, under a condition suitable for hybridizing individual covalently closed circular library molecules (600) to individual immobilized third surface primers thereby immobilizing the plurality of covalently closed circular library molecules (600); and **b)** contacting the plurality of immobilized covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of third surface primers as immobilized amplification primers and the plurality of covalently closed circular library molecules (600) as template molecules, thereby generating a plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0074]** In some embodiment,s the method further comprises: conducting a rolling circle amplification reaction by: **a)** contacting the population of covalently closed circular library molecules (600) that are hybridized to the first splint strand (300) of step (d) or (e) with at least one exonuclease enzyme under a condition suitable to remove the plurality of first splint strands (300) and retaining the population of covalently closed circular library molecules (600); **b)** contacting the retained population of covalently closed circular library molecules (600) with a plurality of soluble amplification primers, a plurality of strand-displacing polymerases, and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction thereby generating a plurality of nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0075]** In some embodiments, the method further comprises: **c)** distributing the plurality of concatemer molecules onto a support having a plurality of the third surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized third surface primers thereby immobilizing the plurality of concatemer molecules; and **d)** contacting the immobilized plurality of concatemer molecules (which are hybridized to a covalently closed

circular library molecule (600)) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0076]** In some embodiments, the method further comprises: conducting a rolling circle amplification reaction by contacting the population of covalently closed circular library molecules (600) each hybridized to the first splint strand (300) of step (d) or (e) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction thereby generating a plurality of concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0077]** In some embodiments, the method further comprises: **c)** distributing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600) onto a support having a plurality of the third surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized third surface primers thereby immobilizing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600); and **d)** contacting the plurality of immobilized concatemer molecules with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0078]** In some embodiments, the method further comprises sequencing the insert region (110) and the first left index sequence (160) and/or the first right index sequence (170) of the plurality of immobilized nucleic acid concatemer molecules, wherein the sequences of the insert region (110) along with the first left index sequence (160) and/or first right index sequence (170) can be used to identify the sample source of the sequence of interest.

**[0079]** In some embodiments, the sequencing reaction comprises: **a)** binding the plurality of immobilized nucleic acid concatemer molecules with a plurality of sequencing primers, a plurality of sequencing polymerases, and a plurality of detectably labeled nucleotide analogs each comprising a 2' or 3' chain terminating moiety, under a condition suitable for

incorporating the detectably labeled nucleotide analogs into the 3' end of the sequencing primers; **b)** detecting the bound detectably labeled nucleotide analog; and **c)** identifying the nucleobase of the detectably labeled nucleotide analog.

**[0080]** In some embodiments, the sequencing reaction employs a two-stage sequencing reaction, wherein the first stage comprises: **a)** binding the plurality of immobilized nucleic acid concatemer molecules with a plurality of sequencing primers, a plurality of a first sequencing polymerase, and a plurality of detectably labeled multivalent molecules, under a condition suitable to bind individual concatemer molecules to a sequencing primer, a first sequencing polymerase, and a detectably labeled multivalent molecule (e.g., forming a plurality of multivalent-complexed polymerases); **b)** detecting the bound detectably labeled multivalent molecule; and **c)** identifying the nucleobase of the detectably labeled multivalent molecule. In some embodiments, the detectably labeled multivalent molecules to the plurality of immobilized nucleic acid concatemer molecules forms at least one avidity complex.

**[0081]** In some embodiments, the method further comprises conducting a second stage which comprises: **d)** removing the plurality of first sequencing polymerases and the plurality of bound multivalent molecules from the plurality of immobilized nucleic acid concatemer molecules and retaining the immobilized concatemer molecules each hybridized to a sequencing primer (nucleic acid duplex); and **e)** contacting the retained immobilized concatemer molecules and hybridized sequencing primers with a plurality of second polymerases and a plurality of nucleotides under a condition suitable for incorporating the nucleotides into the 3' end of the sequencing primers.

**[0082]** The disclosure provides a method for appending additional universal adaptor sequencing to nucleic acid library molecules, comprising: **a)** providing a plurality of nucleic acid library molecules (100) each comprising sequences arranged in a 5' to 3' order: (i) a first left universal adaptor sequence (120), (ii) a second left universal adaptor sequence (140), (iii) a sequence of interest (110), (iv) a second right universal adaptor sequence (150), and (v) a first right universal adaptor sequence (130); **b)** hybridizing individual library molecules of step (a) with a first tailed primer and conducting a first primer extension reaction (or first PCR), wherein the first tailed primers comprise a 5' region carrying a third left universal adaptor sequence and a 3' region that is complementary to at least a portion of the first left universal adaptor sequence (120) of the library molecule (100), wherein the first primer extension reaction (or first PCR) generates a plurality of first modified library molecules each appended at one end with the third left universal adaptor sequence, wherein the third left universal adaptor sequence of the first tailed primers comprise a universal binding sequence

for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer and/or a universal binding sequence for a compaction oligonucleotide; and **c)** hybridizing individual first modified library molecules of step (a) with a second tailed primer and conducting a second primer extension reaction (or second PCR), wherein the second tailed primers comprise a 5' region carrying a third right universal adaptor sequence and a 3' region that is complementary to at least a portion of the first right universal adaptor sequence (130) of the first modified library molecule, wherein the second primer extension reaction (or second PCR) generates a plurality of second modified library molecules each appended at one end with the third right universal adaptor sequence, wherein the third right universal adaptor sequence of the second tailed primers comprise a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer and/or a universal binding sequence for a compaction oligonucleotide.

**[0083]** In some embodiments, the nucleic acid library molecules each comprise sequences arranged in a 5' to 3' order: (i) a first left universal adaptor sequence (120) having a binding sequence for a first surface primer, (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.

**[0084]** In some embodiments, the method further comprises: **d)** hybridizing individual second modified library molecules of step (c) with a third tailed primer and conducting a third primer extension reaction (or third PCR), wherein the third tailed primers comprise a 5' region carrying a fourth left universal adaptor sequence and a 3' region that is complementary to at least a portion of the third left universal adaptor sequence of the second modified library molecule, wherein the third primer extension reaction (or third PCR) generates a plurality of third modified library molecules each appended at one end with the fourth left universal adaptor sequence, wherein the fourth left universal adaptor sequence of the third tailed primers comprise a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer and/or a universal binding sequence for a compaction oligonucleotide; and **e)** hybridizing individual third modified library molecules of step (d) with a fourth tailed primer and conducting a fourth primer extension reaction (or

fourth PCR), wherein the fourth tailed primers comprise a 5' region carrying a fourth right universal adaptor sequence and a 3' region that is complementary to at least a portion of the third right universal adaptor sequence of the third modified library molecule, wherein the fourth primer extension reaction (or fourth PCR) generates a plurality of fourth modified library molecules each appended at one end with the fourth right universal adaptor sequence, wherein the fourth right universal adaptor sequence of the fourth tailed primers comprise a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer and/or a universal binding sequence for a compaction oligonucleotide.

**[0085]** In some embodiments, the method further comprises: **f)** hybridizing individual fourth modified library molecules of step (e) with a fifth tailed primer and conducting a fifth primer extension reaction (or fifth PCR), wherein the fifth tailed primers comprise a 5' region carrying a fifth left universal adaptor sequence and a 3' region that is complementary to at least a portion of the fourth left universal adaptor sequence of the fourth modified library molecule, wherein the fifth primer extension reaction (or fifth PCR) generates a plurality of fifth modified library molecules each appended at one end with the fifth left universal adaptor sequence, wherein the fifth left universal adaptor sequence of the fifth tailed primers comprise a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer and/or a universal binding sequence for a compaction oligonucleotide; and **g)** hybridizing individual fifth modified library molecules of step (f) with a sixth tailed primer and conducting a sixth primer extension reaction (or sixth PCR), wherein the sixth tailed primers comprise a 5' region carrying a sixth right universal adaptor sequence and a 3' region that is complementary to at least a portion of the fourth right universal adaptor sequence of the fifth modified library molecule, wherein the sixth primer extension reaction (or sixth PCR) generates a plurality of sixth modified library molecules each appended at one end with the sixth right universal adaptor sequence, wherein the sixth right universal adaptor sequence of the sixth tailed primers comprise a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer and/or a universal binding sequence for a compaction oligonucleotide.

**[0086]** In some embodiments, the first tailed primer, the third tailed primer and/or the fifth tailed primer further comprises a left index sequence. In some embodiments, the first tailed

primer, the third tailed primer and/or the fifth tailed primer further comprises a left unique identification sequence. In some embodiments, the second tailed primer, the fourth tailed primer and/or the sixth tailed primer further comprises a right index sequence. In some embodiments, the second tailed primer, the fourth tailed primer and/or the sixth tailed primer further comprises a right unique identification sequence. In some embodiments, the appended third left universal adaptor sequence of step (b) can bind a first region (320) of a first splint strand. In some embodiments, the appended third right universal adaptor sequence of step (c) can bind a second region (330) of a first splint strand. In some embodiments, the appended fourth left universal adaptor sequence of step (d) can bind a first region (320) of a first splint strand. In some embodiments, the appended fourth right universal adaptor sequence of step (e) can bind a second region (330) of a first splint strand.

**[0087]** In some embodiments, the appended fifth left universal adaptor sequence of step (f) can bind a first region (320) of a first splint strand. In some embodiments, the appended fifth right universal adaptor sequence of step (g) can bind a second region (330) of a first splint strand.

**[0088]** The disclosure provides a method for forming a plurality of library-splint complexes (500) comprising: **a)** providing a plurality of double-stranded splint adaptors (200) wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the first splint strand (300) comprises regions arranged in a 5' to 3' order a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the second splint strand comprises regions arranged in a 5' to 3' order **(i)** a second sub-region having a universal binding sequence for a fourth surface primer, and **(ii)** a first sub-region having a universal binding sequence for a third surface primer; and **b)** hybridizing the plurality of double-stranded splint adaptors with a plurality of single-stranded nucleic acid library molecules (100) wherein individual library molecules comprise regions arranged in a 5' to 3' order: **(i)** a first left universal adaptor sequence (120) having a binding sequence for a third surface primer; **(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 fourth surface primer, wherein the hybridizing is conducted under a condition suitable to hybridize portions of the first splint strand (300) to portions of the library molecule (100) thereby circularizing the library

molecule to generate a library-splint complex (500), such that the first region (320) of the first splint strand is hybridized to the binding sequence for the third surface primer (120), and the third region (330) of the first splint strand is hybridized to the binding sequence for the fourth surface primer (130), wherein the library-splint complex (500) comprises a first nick between the 5' end of the library molecule and the 3' end of the second splint strand (400), wherein the library-splint complex (500) comprises a second nick between the 5' end of the second splint strand (400) and the 3' end of the library molecule (100), and wherein the first and second nicks are enzymatically ligatable.

**[0089]** In some embodiments, the plurality of single-stranded nucleic acid library molecules (100) further comprise a first left index sequence (160) and/or a first right index sequence (170). In some embodiments, 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).

**[0090]** In some embodiments, the method further comprises: contacting the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a ligase, under a condition suitable to enzymatically ligate the first and second nicks, thereby generating a plurality of covalently closed circular library molecules (600) each hybridized to the first splint strand (300). In some embodiments, the method further comprises: contacting the plurality of covalently closed circular library molecules (600) with at least one exonuclease enzyme to remove the plurality of first splint strands (300) and retaining the plurality of covalently closed circular library molecules (600).

**[0091]** In some embodiments, the individual covalently closed circular library molecules (600) in the plurality comprise a second splint strand region (400) which includes the universal binding sequence for the second surface primer, and the method further comprises: distributing the retained plurality of covalently closed circular library molecules (600) onto a support having a plurality of the second surface primers immobilized on the support, under a condition suitable for hybridizing individual covalently closed circular library molecules (600) to individual immobilized second surface primers thereby immobilizing the plurality of covalently closed circular library molecules (600).

**[0092]** In some embodiments, the method further comprises: contacting the plurality of immobilized covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct 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 (600) as template molecules, thereby generating a plurality of immobilized nucleic acid concatemer molecules. In some embodiments, the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0093]** In some embodiments, the plurality of immobilized second surface capture primers on the support are located at pre-determined or random locations on the support. In some embodiments, the plurality of immobilized second surface capture 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 essentially simultaneously reacted with the reagents in a massively parallel manner, optionally wherein the solution of reagents comprises enzymes, nucleotides and divalent cations. In some embodiments, the density of the plurality of immobilized nucleic acid concatemer molecules on the support is  $10^4 - 10^8$  per  $\text{mm}^2$ .

**[0094]** In some embodiments, the method further comprises: sequencing the plurality of immobilized concatemers, wherein the sequencing comprises: **a)** contacting the plurality of immobilized concatemer molecules with (i) a plurality of sequencing polymerases and (ii) a plurality of the soluble 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 concatemer molecule hybridized to a soluble 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 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.

**[0095]** In some embodiments, the plurality of nucleotides comprise 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. In some embodiments, the plurality of nucleotides comprise one type of nucleotide selected from a

group consisting of dATP, dGTP, dCTP, dTTP and dUTP. In some embodiments, the plurality of nucleotides comprise a mixture of any combination of two or more types of nucleotides selected from a group consisting of dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0096]** In some embodiments, the method further comprises: sequencing the plurality of immobilized concatemers, wherein the sequencing comprises: **a)** contacting the plurality of immobilized concatemer molecules with (i) a plurality of sequencing polymerases and (ii) a plurality of the soluble universal sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each 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 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; and **c)** detecting the plurality of multivalent-complexed polymerases.

**[0097]** In some embodiments, the method further comprises: **d)** 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; **e)** contacting the plurality of the retained nucleic acid duplexes of step d) 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; **f)** 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, 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 e) 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.

**[0098]** In some embodiments, the method further comprises: **g)** detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases. In some embodiments, the method further comprises: **g)** detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases; and **h)** identifying the nucleobases of the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

**[0099]** The disclosure provides a method for sequencing by forming at least one avidity complex, comprising: **a)** binding a first universal sequencing primer, a first sequencing polymerase, and a first detectably labeled multivalent molecule to a first portion of a concatemer molecule of claim 16, thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first sequencing polymerase; **b)** binding a second universal sequencing primer, a second sequencing polymerase, and the first detectably labeled multivalent molecule to a second portion of the same concatemer molecule thereby forming a second binding complex, wherein a second nucleotide unit of the first 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, wherein the first detectably labeled multivalent molecule comprises a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit, wherein the concatemer molecule comprises two or more tandem repeat sequences of a sequence of interest (110) and a universal primer binding site that binds the first and second universal sequencing primers, and 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; **c)** detecting the first and second binding complexes on the same concatemer molecule, and **d)** identifying the first nucleotide unit in the first binding complex thereby determining the sequence of the first portion of the concatemer template molecule, and identifying the second nucleotide unit in the second binding complex thereby determining the sequence of the second portion of the concatemer template molecule.

**[0100]** In some embodiments, the plurality of nucleotide arms attached to the core of the individual multivalent molecules have the same type of a nucleotide unit, and wherein the types of nucleotide unit is selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP. In some embodiments, the plurality of multivalent molecules comprise a mixture of

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

**[0101]** In some embodiments, the plurality of nucleotides comprise 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.

**[0102]** In some embodiments, the plurality of nucleotides comprise one type of nucleotide selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP. In some embodiments, the plurality of nucleotides comprise a mixture of any combination of two or more types of nucleotides selected from a group consisting of dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0103]** In some embodiments, the support comprises a glass or plastic substrate. In some embodiments, the support is passivated with at least one hydrophilic polymer coating having a water contact angle of no more than 45 degrees. In some embodiments, the at least one hydrophilic polymer coating comprises a molecule selected from a group consisting of polyethylene glycol (PEG), poly(vinyl alcohol) (PVA), poly(vinyl pyridine), poly(vinyl pyrrolidone) (PVP), poly(acrylic acid) (PAA), polyacrylamide, poly(N-isopropylacrylamide) (PNIPAM), poly(methyl methacrylate) (PMA), poly(2-hydroxyethyl methacrylate) (HEMA), poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), polyglutamic acid (PGA), poly-lysine, poly-glucoside, streptavidin, and dextran.

## DESCRIPTION OF THE DRAWINGS

**[0104]** 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:

**[0105]** **FIG. 1** is a schematic showing an exemplary linear single stranded library molecule (100) hybridizing with a double-stranded splint molecule (200) thereby circularizing the library molecule to form a library-splint complex (500) with two nicks. The library molecule (100) comprises a sequence of interest (110) flanked on one side by a first left universal

adaptor sequence (120) and flanked on the other side by a first right universal adaptor sequence (130). The double-stranded splint molecule comprises a first splint strand (long strand (300)) hybridized to a second splint strand (short strand (400)). The first splint strand comprises a first region (320) that hybridizes with a sequence on one end of the linear single stranded library molecule, and a second region (330) that hybridizes with a sequence on the other end of the linear single stranded library molecule. The internal region (310) of the first splint strand hybridizes to the second splint strand (400).

**[0106]** FIG. 2 is the same schematic shown in FIG. 1 with more details pertaining to embodiments of the internal region (310) of the first splint strand (300), and the second splint strand (400). The second splint strand (400) includes two sub-regions, where the first sub-region comprises a universal binding sequence for a third surface primer, and the second sub-region comprises a universal binding sequence for a fourth surface primer. The internal region (310) of the first splint strand (300) comprises two sub-regions, where the fourth sub-region hybridizes to the first sub-region of the second splint strand (400), and the fifth sub-region hybridizes to the second sub-region of the second splint strand (400).

**[0107]** FIG. 3 is the same schematic shown in FIG. 1 with more details pertaining to embodiments of the internal region (310) of the first splint strand (300), and the second splint strand (400). The second splint strand (400) includes three sub-regions, where the first sub-region comprises a universal binding sequence for a third surface primer, the second sub-region comprises a universal binding sequence for a fourth surface primer, and the third sub-region comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN). The internal region (310) of the first splint strand (300) comprises three sub-regions, where the fourth sub-region hybridizes to the first sub-region of the second splint strand (400), and the fifth sub-region hybridizes to the second sub-region of the second splint strand (400), and the sixth sub-region hybridizes to the third sub-region of the second splint strand (400).

**[0108]** FIG. 4 is a schematic showing an exemplary linear single stranded library molecule (100) hybridizing with a double-stranded splint molecule (200) thereby circularizing the library molecule to form a library-splint complex (500) with two nicks. The library molecule (100) comprises a sequence of interest (110) flanked on one side by a first left universal adaptor sequence (120) and a second left universal adaptor sequence (140), and flanked on the other side by a second right universal adaptor sequence (150) and a first right universal adaptor sequence (130). The double-stranded splint molecule comprises a first splint strand (long strand (300)) hybridized to a second splint strand (short strand (400)). The first splint

strand comprises a first region (320) that hybridizes with a sequence on one end of the linear single stranded library molecule, and a second region (330) that hybridizes with a sequence on the other end of the linear single stranded library molecule. The internal region (310) of the first splint strand hybridizes to the second splint strand (400).

**[0109] FIG. 5** is a schematic showing an exemplary linear single stranded library molecule (100) hybridizing with a double-stranded splint molecule (200) thereby circularizing the library molecule to form a library-splint complex (500) with two nicks. The library molecule (100) comprises: a first left universal adaptor sequence (120); a first left unique identification sequence (180); a first left index sequence (160); a second left universal adaptor sequence (140); a sequence of interest (110); a second right universal adaptor sequence (150); a first right index sequence (170); and a first right universal adaptor sequence (130). The double-stranded splint molecule comprises a first splint strand (long strand (300)) hybridized to a second splint strand (short strand (400)). The first splint strand comprises a first region (320) that hybridizes with a sequence on one end of the linear single stranded library molecule, and a second region (330) that hybridizes with a sequence on the other end of the linear single stranded library molecule. The internal region (310) of the first splint strand hybridizes to the second splint strand (400).

**[0110] FIG. 6** is a schematic showing an exemplary linear single stranded library molecule (100) hybridizing with a double-stranded splint molecule (200) thereby circularizing the library molecule to form a library-splint complex (500) with two nicks. The library molecule (100) comprises: a first left universal adaptor sequence (120); a first left index sequence (160); a second left universal adaptor sequence (140); a sequence of interest (110); a second right universal adaptor sequence (150); a first right index sequence (170); a first right unique identification sequence (190); and a first right universal adaptor sequence (130). The double-stranded splint molecule comprises a first splint strand (long strand (300)) hybridized to a second splint strand (short strand (400)). The first splint strand comprises a first region (320) that hybridizes with a sequence on one end of the linear single stranded library molecule, and a second region (330) that hybridizes with a sequence on the other end of the linear single stranded library molecule. The internal region (310) of the first splint strand hybridizes to the second splint strand (400).

**[0111] FIG. 7** is a schematic showing an exemplary linear single stranded library molecule (100) hybridizing with a double-stranded splint molecule (200) thereby circularizing the library molecule to form a library-splint complex (500) with two nicks. The library molecule (100) comprises: a first appended left universal adaptor sequence (121); a first left universal

adaptor sequence (120); a first left junction adaptor sequence (125); a first left index sequence (160); a second left junction adaptor sequence (165); a second left universal adaptor sequence (140); a third left junction adaptor sequence (145); a sequence of interest (110); a third right junction adaptor sequence (155); a second right universal adaptor sequence (150); a second right junction adaptor sequence (175); a first right index sequence (170); a first right junction adaptor sequence (135); a first right universal adaptor sequence (130); and a first appended right universal adaptor sequence (131). The double-stranded splint molecule comprises a first splint strand (long strand (300)) hybridized to a second splint strand (short strand (400)). The first splint strand comprises a first region (320) that hybridizes with a sequence on one end of the linear single stranded library molecule, and a second region (330) that hybridizes with a sequence on the other end of the linear single stranded library molecule. The internal region (310) of the first splint strand hybridizes to the second splint strand (400). For the sake of simplicity, the library-splint complex (500) does not show any of the junction adaptor sequences or the appended universal adaptor sequences. The skilled artisan will recognize that the linear library molecule (100) can include any one or any combination of two or more of the junction adaptors, with or without one or both of the appended universal adaptor sequences. The skilled artisan will recognize that the library-splint complex (500) can include any one or any combination of two or more of the junction adaptors, with or without one or both of the appended universal adaptor sequences, that are present in the library molecule (100).

**[0112] FIG. 8** shows three schematics of exemplary covalently closed circular library molecules, each hybridized to a first splint strand (300). The **top** schematic shows a covalently closed circular library molecule (600) having a sequence of interest (110), a first right universal adaptor sequence (130), a second splint strand sequence (400), and a first left universal adaptor sequence (120). The **middle** schematic shows a covalently closed circular library molecule (600) having a sequence of interest (110), a second right universal adaptor sequence (150), a first right universal adaptor sequence (130), a second splint strand sequence (400), a first left universal adaptor sequence (120), and a second left universal adaptor sequence (140). The **bottom** schematic shows a covalently closed circular library molecule (600) having a sequence of interest (110), a second right universal adaptor sequence (150), a first right index sequence (170), a first right universal adaptor sequence (130), a second splint strand sequence (400), a first left universal adaptor sequence (120), a first left unique identification sequence (180), a first left index sequence (160), and a second left universal adaptor sequence (140).

[0113] **FIG. 9** is a schematic showing an exemplary library-splint complex undergoing a ligation reaction to close the nicks to form a covalently closed circular library molecule (600) which is hybridized to a first splint strand (300), where the first splint strand (300) is used as an amplification primer to conduct a rolling circle amplification reaction. The dotted line represents the nascent extension product.

[0114] **FIG. 10** shows the nucleotide sequences of an exemplary double-stranded splint molecule (200) having a first splint strand (300) and a second splint strand (400). The exemplary first splint strand comprises a first region (320; SEQ ID NO:4), a second region (330; SEQ ID NO:5), and an internal region (310) having a fourth sub-region (SEQ ID NO:6) and fifth sub-region (SEQ ID NO:7). The exemplary second splint strand (400) comprises a first sub-region (SEQ ID NO:1) and second sub-region (SEQ ID NO:2).

[0115] **FIG. 11** is a schematic of an exemplary low binding support comprising a glass substrate and alternating layers of hydrophilic coatings which are covalently or non-covalently adhered to the glass, and which further comprises chemically-reactive functional groups that serve as attachment sites for oligonucleotide primers (e.g., capture oligonucleotides and circularization oligonucleotides). In an alternative embodiment, the support can be made of any material such as glass, plastic or a polymer material.

[0116] **FIG. 12** is a schematic of various exemplary configurations of multivalent molecules. **Left:** schematics of multivalent molecules having a starburst or helter-skelter configuration. **Center:** a schematic of a multivalent molecule having a dendrimer configuration. **Right:** a schematic of multiple multivalent molecules formed by reacting streptavidin with 4-arm or 8-arm PEG-NHS with biotin and dNTPs. Nucleotide units are designated 'N', biotin is designated 'B', and streptavidin is designated 'SA'.

[0117] **FIG. 13** is a schematic of an exemplary multivalent molecule comprising a generic core attached to a plurality of nucleotide-arms.

[0118] **FIG. 14** is a schematic of an exemplary multivalent molecule comprising a dendrimer core attached to a plurality of nucleotide-arms.

[0119] **FIG. 15** shows a schematic of an exemplary multivalent molecule comprising a core attached to a plurality of nucleotide-arms, where the nucleotide arms comprise biotin, spacer, linker and a nucleotide unit.

[0120] **FIG. 16** is a schematic of an exemplary nucleotide-arm comprising a core attachment moiety, spacer, linker and nucleotide unit.

[0121] FIG. 17 shows the chemical structure of an exemplary spacer (**top**), and the chemical structures of various exemplary linkers, including an 11-atom Linker, 16-atom Linker, 23-atom Linker and an N3 Linker (**bottom**).

[0122] FIG. 18 shows the chemical structures of various exemplary linkers, including Linkers 1-9.

[0123] FIG. 19 shows the chemical structures of various exemplary linkers joined/attached to nucleotide units.

[0124] FIG. 20 shows the chemical structures of various exemplary linkers joined/attached to nucleotide units.

[0125] FIG. 21 shows the chemical structures of various exemplary linkers joined/attached to nucleotide units.

[0126] FIG. 22 shows the chemical structure of an exemplary biotinylated nucleotide-arm. In this example, the nucleotide unit is connected to the linker via a propargyl amine attachment at the 5 position of a pyrimidine base or the 7 position of a purine base.

[0127] FIGS. 23A-23F is Table 1 (6 sheets) which lists the sequences of exemplary first left index sequences (160) and first right index sequences (170).

[0128] FIG. 24 is a bar graph showing the mean percent recovery of covalently closed circular library molecules using input DNA from various species as determined by qPCR. Lane 1: *Haemophilus influenzae* (38% GC); Lane 2: *E. coli* (51% GC); Lane 3: *Rhodopseudomonas palustris* (65% GC); Lane 4: PhiX; Lane 5: Human; Lane 6: Human exome; Lane 7: Human mRNA. See Examples 1-3.

[0129] FIG. 25 is a bar graph showing the mean polony density obtained by distributing covalently closed circular library molecules onto a support, and performing on-support rolling circle amplification. The covalently closed circular library molecules were prepared from input DNA from various species. Lane 1: cell free DNA; Lane 2: *E. coli*; Lane 3: Human; Lane 4: metagenomic DNA; and Lane 5: PhiX. See Example 4.

[0130] FIG. 26 is a graph showing the nucleotide base diversity of a right sample index sequence (170) including the 3-mer random sequence (NNN). The graph shows a nucleotide diversity of the 3-mer random sequence (NNN) of approximately 30% for A and T base calls, and approximately 20% for C and G base calls.

[0131] FIG. 27 is a graph showing the nucleotide base diversity of a left sample index sequence (160) which lacks a 3-mer random sequence (NNN). The graph shows a nucleotide diversity of approximately 40% for A and T base calls, approximately 15% for C base calls, and approximately 5% for G base calls.

**DETAILED DESCRIPTION**

**[0132]** Throughout this application various publications, patents, and/or patent applications are referenced. The disclosures of the publications, patents and/or patent applications are hereby incorporated by reference in their entireties into this application in order to more fully describe the state of the art to which this disclosure pertains.

**[0133] Definitions:**

**[0134]** The headings provided herein are not limitations of the various aspects of the disclosure, which aspects can be understood by reference to the specification as a whole.

**[0135]** Unless defined otherwise, technical and scientific terms used herein have meanings that are commonly understood by those of ordinary skill in the art unless defined otherwise.

Generally, terminologies pertaining to techniques of molecular biology, nucleic acid chemistry, protein chemistry, genetics, microbiology, transgenic cell production, and hybridization described herein are those well-known and commonly used in the art.

Techniques and procedures described herein are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the instant specification. For example, see Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Third ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 2000). See also Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates (1992). The nomenclatures utilized in connection with, and the laboratory procedures and techniques described herein are those well-known and commonly used in the art.

**[0136]** Unless otherwise required by context herein, singular terms shall include pluralities and plural terms shall include the singular. Singular forms “a”, “an” and “the”, and singular use of any word, include plural referents unless expressly and unequivocally limited on one referent.

**[0137]** It is understood the use of the alternative term (e.g., “or”) is taken to mean either one or both or any combination thereof of the alternatives.

**[0138]** The term “and/or” used herein is to be taken mean specific disclosure of each of the specified features or components with or without the other. For example, the term “and/or” as used in a phrase such as “A and/or B” herein is intended to include: “A and B”; “A or B”; “A” (A alone); and “B” (B alone). In a similar manner, the term “and/or” as used in a phrase such as “A, B, and/or C” is intended to encompass each of the following aspects: “A, B, and

C”; “A, B, or C”; “A or C”; “A or B”; “B or C”; “A and B”; “B and C”; “A and C”; “A” (A alone); “B” (B alone); and “C” (C alone).

**[0139]** As used herein and in the appended claims, terms “comprising”, “including”, “having” and “containing”, and their grammatical variants, as used herein are intended to be non-limiting so that one item or multiple items in a list do not exclude other items that can be substituted or added to the listed items. It is understood that wherever aspects are described herein with the language “comprising,” otherwise analogous aspects described in terms of “consisting of” and/or “consisting essentially of” are also provided.

**[0140]** As used herein, the terms “about” and “approximately” refer to a value or composition that is within an acceptable error range for the particular value or composition as determined by one of ordinary skill in the art, which will depend in part on how the value or composition is measured or determined, i.e., the limitations of the measurement system. For example, “about” or “approximately” can mean within one or more than one standard deviation per the practice in the art. Alternatively, “about” or “approximately” can mean a range of up to 10% (i.e.,  $\pm 10\%$ ) or more depending on the limitations of the measurement system. For example, about 5 mg can include any number between 4.5 mg and 5.5 mg. Furthermore, particularly with respect to biological systems or processes, the terms can mean up to an order of magnitude or up to 5-fold of a value. When particular values or compositions are provided in the instant disclosure, unless otherwise stated, the meaning of “about” or “approximately” should be assumed to be within an acceptable error range for that particular value or composition. Also, where ranges and/or subranges of values are provided, the ranges and/or subranges can include the endpoints of the ranges and/or subranges.

**[0141]** The terms “peptide”, “polypeptide” and “protein” and other related terms used herein are used interchangeably and refer to a polymer of amino acids and are not limited to any particular length. Polypeptides may comprise natural and non-natural amino acids. Polypeptides include recombinant or chemically-synthesized forms. Polypeptides also include precursor molecules that have not yet been subjected to post-translation modification such as proteolytic cleavage, cleavage due to ribosomal skipping, hydroxylation, methylation, lipidation, acetylation, SUMOylation, ubiquitination, glycosylation, phosphorylation and/or disulfide bond formation. These terms encompass native and artificial proteins, protein fragments and polypeptide analogs (such as muteins, variants, chimeric proteins and fusion proteins) of a protein sequence as well as post-translationally, or otherwise covalently or non-covalently, modified proteins.

**[0142]** The term “cellular biological sample” refers to a single cell, a plurality of cells, a tissue, an organ, an organism, or section of any of these cellular biological samples. The cellular biological sample can be extracted (e.g., biopsied) from an organism, or obtained from a cell culture grown in liquid or in a culture dish. The cellular biological sample comprises a sample that is fresh, frozen, fresh frozen, or archived (e.g., formalin-fixed paraffin-embedded; FFPE). The cellular biological sample can be embedded in a wax, resin, epoxy or agar. The cellular biological sample can be fixed, for example in any one or any combination of two or more of acetone, ethanol, methanol, formaldehyde, paraformaldehyde-Triton or glutaraldehyde. The cellular biological sample can be sectioned or non-sectioned. The cellular biological sample can be stained, de-stained or non-stained.

**[0143]** The nucleic acids of interest can be extracted from cells or cellular biological samples using any of a number of techniques known to those of skill in the art. For example, a typical DNA extraction procedure comprises (i) collection of the cell sample or tissue sample from which DNA is to be extracted, (ii) disruption of cell membranes (i.e., cell lysis) to release DNA and other cytoplasmic components, (iii) treatment of the lysed sample with a concentrated salt solution to precipitate proteins, lipids, and RNA, followed by centrifugation to separate out the precipitated proteins, lipids, and RNA, and (iv) purification of DNA from the supernatant to remove detergents, proteins, salts, or other reagents used during the cell membrane lysis. A variety of suitable commercial nucleic acid extraction and purification kits are consistent with the disclosure herein. Examples include, but are not limited to, the QIAamp kits (for isolation of genomic DNA from human samples) and DNAeasy kits (for isolation of genomic DNA from animal or plant samples) from Qiagen (Germantown, MD), or the Maxwell® and ReliaPrep™ series of kits from Promega (Madison, WI).

**[0144]** The term “polymerase” and its variants, as used herein, comprises an enzyme comprising a domain that binds a nucleotide (or nucleoside) where the polymerase can form a complex having a template nucleic acid and a complementary nucleotide. The polymerase can have one or more activities including, but not limited to, base analog detection activities, DNA polymerization activity, reverse transcriptase activity, DNA binding, strand displacement activity, and nucleotide binding and recognition. A polymerase can be any enzyme that can catalyze polymerization of nucleotides (including analogs thereof) into a nucleic acid strand. Typically but not necessarily such nucleotide polymerization can occur in a template-dependent fashion. Typically, a polymerase comprises one or more active sites at which nucleotide binding and/or catalysis of nucleotide polymerization can occur. In some embodiments, a polymerase includes other enzymatic activities, such as for example, 3' to 5'

exonuclease activity or 5' to 3' exonuclease activity. In some embodiments, a polymerase has strand displacing activity. A polymerase can include without limitation naturally occurring polymerases and any subunits and truncations thereof, mutant polymerases, variant polymerases, recombinant, fusion or otherwise engineered polymerases, chemically modified polymerases, synthetic molecules or assemblies, and any analogs, derivatives or fragments thereof that retain the ability to catalyze nucleotide polymerization (e.g., catalytically active fragment). The polymerase includes catalytically inactive polymerases, catalytically active polymerases, reverse transcriptases, and other enzymes comprising a nucleotide binding domain. In some embodiments, a polymerase can be isolated from a cell, or generated using recombinant DNA technology or chemical synthesis methods. In some embodiments, a polymerase can be expressed in prokaryote, eukaryote, viral, or phage organisms. In some embodiments, a polymerase can be post-translationally modified proteins or fragments thereof. A polymerase can be derived from a prokaryote, eukaryote, virus or phage. A polymerase comprises DNA-directed DNA polymerase and RNA-directed DNA polymerase.

**[0145]** The term “strand displacing” refers to the ability of a polymerase to locally separate strands of double-stranded nucleic acids and synthesize a new strand in a template-based manner. Strand displacing polymerases displace a complementary strand from a template strand and catalyze new strand synthesis. Strand displacing polymerases include mesophilic and thermophilic polymerases. Strand displacing polymerases include wild type enzymes, and variants including exonuclease minus mutants, mutant versions, chimeric enzymes and truncated enzymes. Examples of strand displacing polymerases include phi29 DNA polymerase, large fragment of Bst DNA polymerase, large fragment of Bsu DNA polymerase (exo-), Bca DNA polymerase (exo-), Klenow fragment of E. coli DNA polymerase, T5 polymerase, M-MuLV reverse transcriptase, HIV viral reverse transcriptase, Deep Vent DNA polymerase and KOD DNA polymerase. The phi29 DNA polymerase can be wild type phi29 DNA polymerase (e.g., MagniPhi from Expedeon), or variant EquiPhi29 DNA polymerase (e.g., from Thermo Fisher Scientific), or chimeric QualiPhi DNA polymerase (e.g., from 4basebio).

**[0146]** The terms “nucleic acid”, “polynucleotide” and “oligonucleotide” and other related terms used herein are used interchangeably and refer to polymers of nucleotides and are not limited to any particular length. Nucleic acids include recombinant and chemically-synthesized forms. Nucleic acids can be isolated. Nucleic acids include DNA molecules (e.g., cDNA or genomic DNA), RNA molecules (e.g., mRNA), analogs of the DNA or RNA generated using nucleotide analogs (e.g., peptide nucleic acids and non-naturally occurring

nucleotide analogs), and chimeric forms containing DNA and RNA. Nucleic acids can be single-stranded or double-stranded. Nucleic acids comprise polymers of nucleotides, where the nucleotides include natural or non-natural bases and/or sugars. Nucleic acids comprise naturally-occurring internucleosidic linkages, for example phosphodiester linkages. Nucleic acids comprise non-natural internucleoside linkages, including phosphorothioate, phosphorothiolate, or peptide nucleic acid (PNA) linkages. In some embodiments, nucleic acids comprise a one type of polynucleotides or a mixture of two or more different types of polynucleotides.

**[0147]** The term “operably linked” and “operably joined” or related terms as used herein refers to juxtaposition of components. The juxtapositioned components can be linked together covalently. For example, two nucleic acid components can be enzymatically ligated together where the linkage that joins together the two components comprises phosphodiester linkage. A first and second nucleic acid component can be linked together, where the first nucleic acid component can confer a function on a second nucleic acid component. For example, linkage between a primer binding sequence and a sequence of interest forms a nucleic acid library molecule having a portion that can bind to a primer. In another example, a transgene (e.g., a nucleic acid encoding a polypeptide or a nucleic acid sequence of interest) can be ligated to a vector where the linkage permits expression or functioning of the transgene sequence contained in the vector. In some embodiments, a transgene is operably linked to a host cell regulatory sequence (e.g., a promoter sequence) that affects expression of the transgene. In some embodiments, the vector comprises at least one host cell regulatory sequence, including a promoter sequence, enhancer, transcription and/or translation initiation sequence, transcription and/or translation termination sequence, polypeptide secretion signal sequences, and the like. In some embodiments, the host cell regulatory sequence controls expression of the level, timing and/or location of the transgene.

**[0148]** The terms “linked”, “joined”, “attached”, “appended” and variants thereof comprise any type of fusion, bond, adherence or association between any combination of compounds or molecules that is of sufficient stability to withstand use in the particular procedure. The procedure can include but are not limited to: nucleotide binding; nucleotide incorporation; de-blocking (e.g., removal of chain-terminating moiety); washing; removing; flowing; detecting; imaging and/or identifying. Such linkage can comprise, for example, covalent, ionic, hydrogen, dipole-dipole, hydrophilic, hydrophobic, or affinity bonding, bonds or associations involving van der Waals forces, mechanical bonding, and the like. In some embodiments, such linkage occurs intramolecularly, for example linking together the ends of a single-

stranded or double-stranded linear nucleic acid molecule to form a circular molecule. In some embodiments, such linkage can occur between a combination of different molecules, or between a molecule and a non-molecule, including but not limited to: linkage between a nucleic acid molecule and a solid surface; linkage between a protein and a detectable reporter moiety; linkage between a nucleotide and detectable reporter moiety; and the like. Some examples of linkages can be found, for example, in Hermanson, G., “Bioconjugate Techniques”, Second Edition (2008); Aslam, M., Dent, A., “Bioconjugation: Protein Coupling Techniques for the Biomedical Sciences”, London: Macmillan (1998); Aslam, M., Dent, A., “Bioconjugation: Protein Coupling Techniques for the Biomedical Sciences”, London: Macmillan (1998).

**[0149]** The term “primer” and related terms used herein refers to an oligonucleotide that is capable of hybridizing with a DNA and/or RNA polynucleotide template to form a duplex molecule. Primers can be single-stranded along their entire length or have single-stranded and double-stranded portions. Primers comprise natural nucleotides and/or nucleotide analogs. Primers can be recombinant nucleic acid molecules. Primers may have any length, but typically range from 4-50 nucleotides. A typical primer comprises a 5' end and 3' end. The 3' end of the primer can include a 3' OH moiety which serves as a nucleotide polymerization initiation site in a polymerase-catalyzed primer extension reaction. Alternatively, the 3' end of the primer can lack a 3' OH moiety, or can include a terminal 3' blocking group that inhibits nucleotide polymerization in a polymerase-catalyzed reaction. Any one nucleotide, or more than one nucleotide, along the length of the primer can be labeled with a detectable reporter moiety. A primer can be in solution (e.g., a soluble primer) or can be immobilized to a support (e.g., a capture primer).

**[0150]** The term “template nucleic acid”, “template polynucleotide”, “target nucleic acid” “target polynucleotide”, “template strand” and other variations refer to a nucleic acid strand that serves as the basis nucleic acid molecule for any of the amplification and/or sequencing methods describe herein. The template nucleic acid can be single-stranded or double-stranded, or the template nucleic acid can have single-stranded or double-stranded portions. The template nucleic acid can be obtained from a naturally-occurring source, recombinant form, or chemically synthesized to include any type of nucleic acid analog. The template nucleic acid can be linear, concatemeric, circular, or other forms.

**[0151]** The term “adaptor” and related terms refers to oligonucleotides that can be operably linked (appended) to a target polynucleotide, where the adaptor confers a function to the co-joined adaptor-target molecule. Adaptors comprise DNA, RNA, chimeric DNA/RNA, or

analogous thereof. Adaptors can include at least one ribonucleoside residue. Adaptors can be single-stranded, double-stranded, or have single-stranded and/or double-stranded portions. Adaptors can be configured to be linear, stem-looped, hairpin, or Y-shaped forms. Adaptors can be any length, including 4-100 nucleotides or longer. Adaptors can have blunt ends, overhang ends, or a combination of both. Overhang ends include 5' overhang and 3' overhang ends. The 5' end of a single-stranded adaptor, or one strand of a double-stranded adaptor, can have a 5' phosphate group or lack a 5' phosphate group. Adaptors can include a 5' tail that does not hybridize to a target polynucleotide (e.g., tailed adaptor), or adaptors can be non-tailed. At least a portion of the adaptors comprise a known and pre-determined sequence. An adaptor can include a sequence that is complementary to at least a portion of a primer, such as an amplification primer, a sequencing primer, or a capture primer (e.g., soluble or immobilized capture primers). Adaptors can include a random sequence or degenerate sequence. Adaptors can include at least one inosine residue. Adaptors can include at least one phosphorothioate, phosphorothiolate and/or phosphoramidate linkage. Adaptors can include at least one barcode/index sequence which can be used to distinguish polynucleotides (e.g., insert sequences) from different sample sources in a multiplex assay. Adaptors can include at least one unique identification sequence (e.g., a molecular tag) that can be used to uniquely identify a nucleic acid molecule to which the adaptor is appended. In some embodiments, the unique identification sequence comprises 2-12 or more nucleotides having a known sequence. For example, the unique identification sequence comprises a known random sequence where a nucleotide at each position is randomly selected from nucleotides having a base A, G, C, T or U. Adaptors can include at least one restriction enzyme recognition sequence, including any one or any combination of two or more selected from a group consisting of type I, type II, type III, type IV, type Hs or type IIB.

**[0152]** The term “universal sequence” and related terms refers to a sequence in a nucleic acid molecule that is common among two or more polynucleotide molecules. For example, an adaptor having a universal sequence can be operably joined to a plurality of polynucleotides so that the population of co-joined molecules carry the same universal adaptor sequence. Examples of universal adaptor sequences include an amplification primer sequence, a sequencing primer sequence or a capture primer sequence (e.g., soluble or immobilized capture primers).

**[0153]** When used in reference to nucleic acid molecules, the terms “hybridize” or “hybridizing” or “hybridization” or other related terms refers to hydrogen bonding between two different nucleic acids to form a duplex nucleic acid. Hybridization also includes

hydrogen bonding between two different regions of a single nucleic acid molecule to form a self-hybridizing molecule having a duplex region. Hybridization can comprise Watson-Crick or Hoogsteen binding to form a duplex double-stranded nucleic acid, or a double-stranded region within a nucleic acid molecule. The double-stranded nucleic acid, or the two different regions of a single nucleic acid, may be wholly complementary, or partially complementary. Complementary nucleic acid strands need not hybridize with each other across their entire length. The complementary base pairing can be the standard A-T or C-G base pairing, or can be other forms of base-pairing interactions. Duplex nucleic acids can include mismatched base-paired nucleotides.

**[0154]** When used in reference to nucleic acids, the terms “extend”, “extending”, “extension” and other variants, refers to incorporation of one or more nucleotides into a nucleic acid molecule. Nucleotide incorporation comprises polymerization of one or more nucleotides into the terminal 3' OH end of a nucleic acid strand, resulting in extension of the nucleic acid strand. Nucleotide incorporation can be conducted with natural nucleotides and/or nucleotide analogs. Typically, but not necessarily, nucleotide incorporation occurs in a template-dependent fashion. Any suitable method of extending a nucleic acid molecule may be used, including primer extension catalyzed by a DNA polymerase or RNA polymerase.

**[0155]** The term “nucleotides” and related terms refers to a molecule comprising an aromatic base, a five carbon sugar (e.g., ribose or deoxyribose), and at least one phosphate group. Canonical or non-canonical nucleotides are consistent with use of the term. In some embodiments, the nucleotide comprises a monophosphate, diphosphate, or triphosphate, or corresponding phosphate analog. The term “nucleoside” refers to a molecule comprising an aromatic base and a sugar. Nucleotides and nucleosides can be non-labeled or labeled with a detectable reporter moiety.

**[0156]** Nucleotides (and nucleosides) typically comprise a hetero cyclic base including substituted or unsubstituted nitrogen-containing parent heteroaromatic ring which are commonly found in nucleic acids, including naturally-occurring, substituted, modified, or engineered variants, or analogs of the same. The base of a nucleotide (or nucleoside) is capable of forming Watson-Crick and/or Hoogsteen hydrogen bonds with an appropriate complementary base. Exemplary bases include, but are not limited to, purines and pyrimidines such as: 2-aminopurine, 2,6-diaminopurine, adenine (A), ethenoadenine, N<sup>6</sup>-Δ<sup>2</sup>-isopentenyladenine (6iA), N<sup>6</sup>-Δ<sup>2</sup>-isopentenyl-2-methylthioadenine (2ms6iA), N<sup>6</sup>-methyladenine, guanine (G), isoguanine, N<sup>2</sup>-dimethylguanine (dmG), 7-methylguanine (7mG), 2-thiopyrimidine, 6-thioguanine (6sG), hypoxanthine and O<sup>6</sup>-methylguanine; 7-

deaza-purines such as 7-deazaadenine (7-deaza-A) and 7-deazaguanine (7-deaza-G); pyrimidines such as cytosine (C), 5-propynylcytosine, isocytosine, thymine (T), 4-thiothymine (4sT), 5,6-dihydrothymine, O<sup>4</sup>-methylthymine, uracil (U), 4-thiouracil (4sU) and 5,6-dihydrouracil (dihydrouracil; D); indoles such as nitroindole and 4-methylindole; pyrroles such as nitropyrrole; nebularine; inosines; hydroxymethylcytosines; 5-methylcytosines; base (Y); as well as methylated, glycosylated, and acylated base moieties; and the like. Additional exemplary bases can be found in Fasman, 1989, in “Practical Handbook of Biochemistry and Molecular Biology”, pp. 385-394, CRC Press, Boca Raton, Fla.

**[0157]** Nucleotides (and nucleosides) typically comprise a sugar moiety, such as carbocyclic moiety (Ferraro and Gotor 2000 Chem. Rev. 100: 4319-48), acyclic moieties (Martinez, et al., 1999 Nucleic Acids Research 27: 1271-1274; Martinez, et al., 1997 Bioorganic & Medicinal Chemistry Letters vol. 7: 3013-3016), and other sugar moieties (Joeng, et al., 1993 J. Med. Chem. 36: 2627-2638; Kim, et al., 1993 J. Med. Chem. 36: 30-7; Eschenmosser 1999 Science 284:2118-2124; and U.S. Pat. No. 5,558,991). The sugar moiety comprises: ribosyl; 2'-deoxyribosyl; 3'-deoxyribosyl; 2',3'-dideoxyribosyl; 2',3'-didehydrideoxyribosyl; 2'-alkoxyribosyl; 2'-azidoribosyl; 2'-aminoribosyl; 2'-fluororibosyl; 2'-mercaptoriboxyl; 2'-alkylthioribosyl; 3'-alkoxyribosyl; 3'-azidoribosyl; 3'-aminoribosyl; 3'-fluororibosyl; 3'-mercaptoriboxyl; 3'-alkylthioribosyl carbocyclic; acyclic or other modified sugars.

**[0158]** In some embodiments, nucleotides comprise a chain of one, two or three phosphorus atoms where the chain is typically attached to the 5' carbon of the sugar moiety via an ester or phosphoramidate linkage. In some embodiments, the nucleotide is an analog having a phosphorus chain in which the phosphorus atoms are linked together with intervening O, S, NH, methylene or ethylene. In some embodiments, the phosphorus atoms in the chain include substituted side groups including O, S or BH<sub>3</sub>. In some embodiments, the chain includes phosphate groups substituted with analogs including phosphoramidate, phosphorothioate, phosphordithioate, and O-methylphosphoroamidite groups.

**[0159]** The term “rolling circle amplification” generally refers to an amplification method that employs a circularized nucleic acid template molecule containing a target sequence of interest, an amplification primer binding sequence, and optionally one or more adaptor sequences such as a sequencing primer binding sequence and/or a sample index sequence. The rolling circle amplification reaction can be conducted under isothermal amplification conditions, and includes the circularized nucleic acid template molecule, an amplification primer, a strand-displacing polymerase and a plurality of nucleotides, to generate a

concatemer containing tandem repeat sequences of the circular template molecule and any adaptor sequences present in the original circularized nucleic acid template molecule. The concatemer can self-collapse to form a nucleic acid nanoball. The shape and size of the nanoball can be further compacted by including a pair of inverted repeat sequences in the circular template molecule, or by conducting the rolling circle amplification reaction with one or more compaction oligonucleotides. One of the advantages of using rolling circle amplification to generate clonal amplicons for a sequencing workflow is that the repeat copies of the target sequence in the nanoball can be simultaneously sequenced to increase signal intensity. In some embodiments, the rolling circle amplification reaction can be conducted in the presence of a plurality of compaction oligonucleotides having at least four consecutive guanines. The rolling circle amplification reaction generates concatemers comprising repeat copies of the universal binding sequence for the compaction oligonucleotide. At least one compaction oligonucleotide can form a guanine tetrad and hybridize to the universal binding sequences for the compaction oligonucleotide, and the resulting concatemer can fold to form an intramolecular G-quadruplex structure. The concatemers can self-collapse to form compact nanoballs. Formation of the guanine tetrads and G-quadruplexes in the nanoballs may increase the stability of the nanoballs to retain their compact size and shape which can withstand repeated flows of reagents for conducting any of the sequencing workflows described herein.

**[0160]** When used in reference to nucleic acids, the terms “amplify”, “amplifying”, “amplification”, and other related terms include producing multiple copies of an original polynucleotide template molecule, where the copies comprise a sequence that is complementary to the template sequence, and/or the copies comprise a sequence that is the same as the template sequence. In some embodiments, the copies comprise a sequence that is substantially identical to a template sequence, and/or is substantially identical to a sequence that is complementary to the template sequence.

**[0161]** The term “reporter moiety”, “reporter moieties” or related terms refers to a compound that generates, or causes to generate, a detectable signal. A reporter moiety is sometimes called a “label”. Any suitable reporter moiety may be used, including luminescent, photoluminescent, electroluminescent, bioluminescent, chemiluminescent, fluorescent, phosphorescent, chromophore, radioisotope, electrochemical, mass spectrometry, Raman, hapten, affinity tag, atom, or an enzyme. A reporter moiety generates a detectable signal resulting from a chemical or physical change (e.g., heat, light, electrical, pH, salt concentration, enzymatic activity, or proximity events). A proximity event includes two

reporter moieties approaching each other, or associating with each other, or binding each other. It is well known to one skilled in the art to select reporter moieties so that each absorbs excitation radiation and/or emits fluorescence at a wavelength distinguishable from the other reporter moieties to permit monitoring the presence of different reporter moieties in the same reaction or in different reactions. Two or more different reporter moieties can be selected having spectrally distinct emission profiles, or having minimal overlapping spectral emission profiles. Reporter moieties can be linked (e.g., operably linked) to nucleotides, nucleosides, nucleic acids, enzymes (e.g., polymerases or reverse transcriptases), or support (e.g., surfaces).

**[0162]** A fluorescent reporter moiety (or label) comprises a fluorescent label or a fluorophore. Exemplary fluorescent moieties which may serve as fluorescent labels or fluorophores include, but are not limited to fluorescein and fluorescein derivatives such as carboxyfluorescein, tetrachlorofluorescein, hexachlorofluorescein, carboxynaphthofluorescein, fluorescein isothiocyanate, NHS-fluorescein, iodoacetamidofluorescein, fluorescein maleimide, SAMSA-fluorescein, fluorescein thiosemicarbazide, carbohydrazinomethylthioacetyl-amino fluorescein, rhodamine and rhodamine derivatives such as TRITC, TMR, lissamine rhodamine, Texas Red, rhodamine B, rhodamine 6G, rhodamine 10, NHS-rhodamine, TMR-iodoacetamide, lissamine rhodamine B sulfonyl chloride, lissamine rhodamine B sulfonyl hydrazine, Texas Red sulfonyl chloride, Texas Red hydrazide, coumarin and coumarin derivatives such as AMCA, AMCA-NHS, AMCA-sulfo-NHS, AMCA-HPDP, DCIA, AMCE-hydrazide, BODIPY and derivatives such as BODIPY FL C3-SE, BODIPY 530/550 C3, BODIPY 530/550 C3-SE, BODIPY 530/550 C3 hydrazide, BODIPY 493/503 C3 hydrazide, BODIPY FL C3 hydrazide, BODIPY FL IA, BODIPY 530/551 IA, Br-BODIPY 493/503, Cascade Blue and derivatives such as Cascade Blue acetyl azide, Cascade Blue cadaverine, Cascade Blue ethylenediamine, Cascade Blue hydrazide, Lucifer Yellow and derivatives such as Lucifer Yellow iodoacetamide, Lucifer Yellow CH, cyanine and derivatives such as indolium based cyanine dyes, benzo-indolium based cyanine dyes, pyridium based cyanine dyes, thiazolium based cyanine dyes, quinolinium based cyanine dyes, imidazolium based cyanine dyes, Cy 3, Cy5, lanthanide chelates and derivatives such as BCPDA, TBP, TMT, BHHCT, BCOT, Europium chelates, Terbium chelates, Alexa Fluor dyes, DyLight dyes, Atto dyes, LightCycler Red dyes, CAL Flour dyes, JOE and derivatives thereof, Oregon Green dyes, WellRED dyes, IRD dyes, phycoerythrin and phycobilin dyes, Malachite green, stilbene, DEG dyes, NR dyes, near-infrared dyes and others known in the art such as those described in Haugland, Molecular Probes Handbook,

(Eugene, Oreg.) 6th Edition; Lakowicz, Principles of Fluorescence Spectroscopy, 2nd Ed., Plenum Press New York (1999), or Hermanson, Bioconjugate Techniques, 2nd Edition, or derivatives thereof, or any combination thereof. Cyanine dyes may exist in either sulfonated or non-sulfonated forms, and consist of two indolenin, benzo-indolium, pyridium, thiozolium, and/or quinolinium groups separated by a polymethine bridge between two nitrogen atoms. Commercially available cyanine fluorophores include, for example, Cy3, (which may comprise 1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-2-(3-{1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-3,3-dimethyl-1,3-dihydro-2H-indol-2-ylidene}prop-1-en-1-yl)-3,3-dimethyl-3H-indolium or 1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-2-(3-{1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-3,3-dimethyl-5-sulfo-1,3-dihydro-2H-indol-2-ylidene}prop-1-en-1-yl)-3,3-dimethyl-3H-indolium-5-sulfonate), Cy5 (which may comprise 1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-2-((1E,3E)-5-((E)-1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium or 1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-2-((1E,3E)-5-((E)-1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate), and Cy7 (which may comprise 1-(5-carboxypentyl)-2-[(1E,3E,5E,7Z)-7-(1-ethyl-1,3-dihydro-2H-indol-2-ylidene)hepta-1,3,5-trien-1-yl]-3H-indolium or 1-(5-carboxypentyl)-2-[(1E,3E,5E,7Z)-7-(1-ethyl-5-sulfo-1,3-dihydro-2H-indol-2-ylidene)hepta-1,3,5-trien-1-yl]-3H-indolium-5-sulfonate), where “Cy” stands for 'cyanine', and the first digit identifies the number of carbon atoms between two indolenine groups. Cy2 which is an oxazole derivative rather than indolenin, and the benzo-derivatized Cy3.5, Cy5.5 and Cy7.5 are exceptions to this rule.

**[0163]** In some embodiments, the reporter moiety can be a fluorescence resonance energy transfer (FRET) pair, such that multiple classifications can be performed under a single excitation and imaging step. As used herein, FRET may comprise excitation exchange (Forster) transfers, or electron-exchange (Dexter) transfers.

**[0164]** The term “support” as used herein refers to a substrate that is designed for deposition of biological molecules or biological samples for assays and/or analyses. Examples of biological molecules to be deposited onto a support include nucleic acids (e.g., DNA, RNA), polypeptides, saccharides, lipids, a single cell or multiple cells. Examples of biological samples include but are not limited to saliva, phlegm, mucus, blood, plasma, serum, urine, stool, sweat, tears and fluids from tissues or organs.

**[0165]** In some embodiments, the support is solid, semi-solid, or a combination of both. In some embodiments, the support is porous, semi-porous, non-porous, or any combination of porosity. In some embodiments, the support can be substantially planar, concave, convex, or any combination thereof. In some embodiments, the support can be cylindrical, for example comprising a capillary or interior surface of a capillary.

**[0166]** In some embodiments, the surface of the support can be substantially smooth. In some embodiments, the support can be regularly or irregularly textured, including bumps, etched, pores, three-dimensional scaffolds, or any combination thereof.

**[0167]** In some embodiments, the support comprises a bead having any shape, including spherical, hemi-spherical, cylindrical, barrel-shaped, toroidal, disc-shaped, rod-like, conical, triangular, cubical, polygonal, tubular or wire-like.

**[0168]** The support can be fabricated from any material, including but not limited to glass, fused-silica, silicon, a polymer (e.g., polystyrene (PS), macroporous polystyrene (MPPS), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET)), or any combination thereof. Various compositions of both glass and plastic substrates are contemplated.

**[0169]** The present disclosure provides a plurality (e.g., two or more) of nucleic acid template molecules immobilized to a support. In some embodiments, the immobilized plurality of nucleic acid template molecules have the same sequence or have different sequences. In some embodiments, individual nucleic acid template molecules in the plurality of nucleic acid template molecules are immobilized to a different site on the support. In some embodiments, two or more individual nucleic acid template molecules in the plurality of nucleic acid templates are immobilized to a site on the support.

**[0170]** The term “array” refers to a support comprising a plurality of sites located at pre-determined locations on the support to form an array of sites. The sites can be discrete and separated by interstitial regions. In some embodiments, the pre-determined sites on the support can be arranged in one dimension in a row or a column, or arranged in two dimensions in rows and columns. In some embodiments, the plurality of pre-determined sites is arranged on the support in an organized fashion. In some embodiments, the plurality of pre-determined sites is arranged in any organized pattern, including rectilinear, hexagonal patterns, grid patterns, patterns having reflective symmetry, patterns having rotational symmetry, or the like. The pitch between different pairs of sites can be that same or can vary. In some embodiments, the support comprises at least  $10^2$  sites, at least  $10^3$  sites, at least  $10^4$

sites, at least  $10^5$  sites, at least  $10^6$  sites, at least  $10^7$  sites, at least  $10^8$  sites, at least  $10^9$  sites, at least  $10^{10}$  sites, at least  $10^{11}$  sites, at least  $10^{12}$  sites, at least  $10^{13}$  sites, at least  $10^{14}$  sites, at least  $10^{15}$  sites, or more, where the sites are located at pre-determined locations on the support. In some embodiments, a plurality of pre-determined sites on the support (e.g.,  $10^2 - 10^{15}$  sites or more) are immobilized with nucleic acid template molecules to form a nucleic acid template array. In some embodiments, the nucleic acid template molecules that are immobilized at a plurality of pre-determined sites by hybridization to immobilized surface capture primers, or the nucleic acid template molecules are covalently attached to the surface capture primers. In some embodiments, the nucleic acid template molecules that are immobilized at a plurality of pre-determined sites, for example immobilized at  $10^2 - 10^{15}$  sites or more. In some embodiments, the immobilized nucleic acid template molecules are clonally-amplified to generate immobilized nucleic acid clusters at the plurality of pre-determined sites. In some embodiments, individual immobilized nucleic acid clusters comprise linear clusters, or comprise single-stranded or double-stranded concatemers.

**[0171]** In some embodiments, a support comprising a plurality of sites located at random locations on the support is referred to herein as a support having randomly located sites thereon. The location of the randomly located sites on the support are not pre-determined. The plurality of randomly-located sites is arranged on the support in a disordered and/or unpredictable fashion. In some embodiments, the support comprises at least  $10^2$  sites, at least  $10^3$  sites, at least  $10^4$  sites, at least  $10^5$  sites, at least  $10^6$  sites, at least  $10^7$  sites, at least  $10^8$  sites, at least  $10^9$  sites, at least  $10^{10}$  sites, at least  $10^{11}$  sites, at least  $10^{12}$  sites, at least  $10^{13}$  sites, at least  $10^{14}$  sites, at least  $10^{15}$  sites, or more, where the sites are randomly located on the support. In some embodiments, a plurality of randomly located sites on the support (e.g.,  $10^2 - 10^{15}$  sites or more) are immobilized with nucleic acid template molecules. In some embodiments, the nucleic acid template molecules are immobilized at a plurality of randomly located sites by hybridization to immobilized surface capture primers, or the nucleic acid template molecules are covalently attached to the surface capture primers. In some embodiments, the nucleic acid templates that are immobilized at a plurality of randomly located sites, for example immobilized at  $10^2 - 10^{15}$  sites or more. In some embodiments, the immobilized nucleic acid templates are clonally-amplified to generate immobilized nucleic acid clusters at the plurality of randomly located sites. In some embodiments, individual immobilized nucleic acid clusters comprise linear clusters, or comprise single-stranded or double-stranded concatemers.

**[0172]** In some embodiments, the plurality of immobilized surface capture primers on the support (e.g., located at pre-determined or random locations on the support) are in fluid communication with each other to permit flowing a solution of reagents (e.g., nucleic acid template molecules, soluble primers, enzymes, nucleotides, divalent cations, buffers, and the like) onto the support so that the plurality of immobilized surface capture primers on the support can be essentially simultaneously reacted with the reagents in a massively parallel manner. In some embodiments, the fluid communication of the plurality of immobilized surface capture primers can be used to conduct nucleic acid amplification reactions (e.g., RCA, MDA, PCR and bridge amplification) essentially simultaneously on the plurality of immobilized surface capture primers.

**[0173]** In some embodiments, the plurality of immobilized nucleic acid clusters on the support are in fluid communication with each other to permit flowing a solution of reagents (e.g., enzymes, nucleotides, divalent cations, and the like) onto the support so that the plurality of immobilized nucleic acid clusters on the support can be essentially simultaneously reacted with the reagents in a massively parallel manner. In some embodiments, the fluid communication of the plurality of immobilized nucleic acid clusters can be used to conduct nucleotide binding assays and/or conduct nucleotide polymerization reactions (e.g., primer extension or sequencing) essentially simultaneously on the plurality of immobilized nucleic acid clusters, and optionally to conduct detection and imaging for massively parallel sequencing.

**[0174]** In some embodiments, the term “immobilized” and related terms refer to nucleic acid molecules that are attached to a support through covalent bond or non-covalent interaction, or attached to a coating on the support, or buried within a matrix formed by a coating on the support, where the nucleic acid molecules include surface capture primers, nucleic acid template molecules and extension products of capture primers. Extension products of capture primers includes nucleic acid concatemers (e.g., nucleic acid clusters). The nucleic acid molecules can be immobilized at pre-determined or random locations on the support. The nucleic acid molecules can be immobilized at pre-determined or random locations on or within a coating passivated on the support.

**[0175]** In some embodiments, the term “immobilized” and related terms refer to enzymes (e.g., polymerases) that are attached to a support through covalent bond or non-covalent interaction, or attached to a coating on the support, or buried within a matrix formed by a coating on the support. The enzymes can be immobilized at pre-determined or random

locations on the support. The enzymes can be immobilized at pre-determined or random locations on or within a coating passivated on the support.

**[0176]** In some embodiments, one or more nucleic acid template molecules are immobilized on the support, for example immobilized at the sites on the support. In some embodiments, the one or more nucleic acid template molecules are clonally-amplified. In some embodiments, the one or more nucleic acid template molecules are clonally-amplified off the support (e.g., in-solution) and then deposited onto the support and immobilized on the support. In some embodiments, the clonal amplification reaction of the one or more nucleic acid template molecules is conducted on the support resulting in immobilization on the support. In some embodiments, the one or more nucleic acid template molecules are clonally-amplified (e.g., in solution or on the support) using a nucleic acid amplification reaction, including any one or any combination of: polymerase chain reaction (PCR), multiple displacement amplification (MDA), transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), real-time SDA, bridge amplification, isothermal bridge amplification, rolling circle amplification (RCA), circle-to-circle amplification, helicase-dependent amplification, recombinase-dependent amplification, and/or single-stranded binding (SSB) protein-dependent amplification.

**[0177]** The term “surface primer” and related terms refers to single-stranded oligonucleotides that are immobilized to a support and comprise a sequence that can hybridize to at least a portion of a nucleic acid template molecule. Surface capture primers can be used to immobilize template molecules to a support via hybridization. Surface capture primers can be immobilized to a support in a manner that resists primer removal during flowing, washing, aspirating, and changes in temperature, pH, salts, chemical and/or enzymatic conditions. Typically, but not necessarily, the 5' end of a surface capture primer can be immobilized to a support or to a coating on the support (or embedded in a coating on the support). Alternatively, an interior portion or the 3' end of a surface capture primer can be immobilized to a support.

**[0178]** The sequence of surface capture primers can be wholly or partially complementary along their length to at least a portion of the nucleic acid template molecule. A support can include a plurality of immobilized surface capture primers having the same sequence, or having two or more different sequences. Surface capture primers can be any length, for example 4-50 nucleotides, or 50-100 nucleotides, or 100-150 nucleotides, or longer lengths.

**[0179]** A surface capture primer can have a terminal 3' nucleotide having a sugar 3' OH moiety which is extendible for nucleotide polymerization (e.g., polymerase catalyzed polymerization). A surface capture primer can have a terminal 3' nucleotide having the 3' sugar position linked to a chain-terminating moiety that inhibits nucleotide polymerization. The 3' chain-terminating moiety can be removed (e.g., de-blocked) to convert the 3' end to an extendible 3' OH end using a de-blocking agent. Examples of chain terminating moieties include alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group. Azide type chain terminating moieties including azide, azido and azidomethyl groups. Examples of de-blocking agents include a phosphine compound, such as Tris(2-carboxyethyl)phosphine (TCEP) and bis-sulfo triphenyl phosphine (BS-TPP), for chain-terminating groups azide, azido and azidomethyl groups. Examples of de-blocking agents include tetrakis(triphenylphosphine)palladium(0) ( $\text{Pd(PPh}_3)_4$ ) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ), for chain-terminating groups alkyl, alkenyl, alkynyl and allyl. Examples of a de-blocking agent includes Pd/C for chain-terminating groups aryl and benzyl. Examples of de-blocking agents include phosphine, beta-mercaptoethanol or dithiothritol (DTT), for chain-terminating groups amine, amide, keto, isocyanate, phosphate, thio and disulfide. Examples of de-blocking agents include potassium carbonate ( $\text{K}_2\text{CO}_3$ ) in MeOH, triethylamine in pyridine, and Zn in acetic acid (AcOH), for carbonate chain-terminating groups. Examples of de-blocking agents include tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, and triethylamine trihydrofluoride, for chain-terminating groups urea and silyl.

**[0180]** The term “sequencing” and related terms refers to a method for obtaining nucleotide sequence information from a nucleic acid molecule, typically by determining the identity of at least some nucleotides (including their nucleobase components) within the nucleic acid molecule. In some embodiments, the sequence information of a given region of a nucleic acid molecule includes identifying each and every nucleotide within a region that is sequenced. In some embodiments, sequencing information determines only some of the nucleotides a region, while the identity of some nucleotides remains undetermined or incorrectly determined. Any suitable method of sequencing may be used. In an exemplary embodiment, sequencing can include label-free or ion based sequencing methods. In some embodiments, sequencing can include labeled or dye-containing nucleotide or fluorescent based nucleotide sequencing methods. In some embodiments, sequencing can include polony-based

sequencing or bridge sequencing methods. In some embodiments, the sequencing employs polymerases and multivalent molecules for generating at least one avidity complex, wherein individual multivalent molecules comprise a plurality of nucleotide units tethered to a core. In some embodiments, the sequencing employs polymerases and free nucleotides for performing sequencing-by-synthesis. In some embodiments, the sequencing employs a ligase enzyme and a plurality of sequence-specific oligonucleotides for performing sequence-by-ligation.

### **Introduction: Double-Stranded Splint Adaptors**

**[0181]** The present disclosure provides compositions comprising nucleic acid double-stranded splint adaptors, including kits, and methods that employ the double-stranded splint adaptors.

**[0182]** The double-stranded splint adaptors (200) can be used in a one-pot, multi-enzyme reaction to introduce one or more new adaptor sequences into a library molecule. The double-stranded splint adaptor (200) comprises a first splint strand (long splint strand (300)) and a second splint strand (short splint strand (400)), where the first and second splint strands are hybridized together to form the double-stranded splint adaptor (200) having a double-stranded region and two flanking single-stranded regions (e.g., see FIGS. 1-7). The second splint strand (400) carries the new adaptor sequence(s) to be introduced, such as for example a new universal binding sequence and/or a new index sequence. The first splint strand comprises a first region (320), an internal region (310), and a second region (330). The internal region of the first splint strand (310) is hybridized to the second splint strand (400). The two flanking single-stranded regions of the double-stranded splinted adaptor (e.g., (320) and (330)) are designed to hybridize to universal adaptor sequences at the ends of a single-stranded linear library molecule (100) having a sequence of interest (110). For example, the first region of the first splint strand (320) is hybridized to one end of the library molecule, and the second region of the first splint strand (330) is hybridized to the other end of the library molecule, thereby circularizing the library molecule to generate a library-splint complex (500) which includes two nicks (e.g., see FIGS. 1-7). The nicks can be enzymatically ligated to generate a covalently closed circular molecule (600) in which the second splint strand (400) is covalently joined at both ends to the library molecule, thereby introducing the new adaptor sequences into the library molecule.

**[0183]** Thus, the double-stranded splint adaptors and the methods described herein, can be used to convert any linear library molecule into a covalently closed circular molecule which

can be used for a different workflow such as a different massively parallel sequencing platform. The double-stranded splint adaptors offer flexibility because the two flanking single-stranded regions (e.g., (320) and (330)), and the second splint strand (400) can be designed to include any combination of universal adaptor sequences. For example, the two flanking single-stranded regions (e.g., (320) and (330)) can comprise universal binding sequences (or complementary sequences thereof) for P5 and P7 sequences which bind to surface primers immobilized on a support (e.g., flow cell), where P5 and P7 sequences are typically used to construct library molecules for an Illumina sequencing platform. The second splint strand (400) can include at least one new universal adaptor sequence (e.g., a new surface primer sequence) that is not found on an Illumina sequencing platform, thereby permitting use of the covalently closed circular molecule (600) on a non-Illumina sequencing platform.

**[0184]** The methods described herein also offer the advantage of employing a ligation reaction rather than a gap fill-in reaction to introduce the new adaptor sequences. The ligation reaction gives a high efficiency circularization with as little as 0.25 pmol library molecules.

**[0185]** The methods described herein can be performed manually or adapted for automation because the annealing and multi-enzyme reactions can be conducted in a single reaction vessel (one-pot) by combining some enzymatic reactions (e.g., phosphorylation and ligation) and by adding subsequent enzymes (e.g., exonucleases) without intervening alcohol precipitations or organic extractions.

### **Double-Stranded Splint Adaptors**

**[0186]** The present disclosure provides nucleic acid double-stranded splint adaptors (200), comprising: **(i)** a first splint strand (long splint strand (300)) which is hybridized to **(ii)** a second splint strand (short splint strand (400)) (e.g., see FIGS. 1-7). The first splint strand comprises a first region (320), an internal region (310), and a second region (330). The internal region of the first splint strand (310) is hybridized to the second splint strand (400) to form a double-stranded splint adaptor (200) having a double-stranded region and two flanking single-stranded regions. The two flanking single-stranded regions of the double-stranded splint adaptor (200) are designed to hybridize to the end sequences of a linear nucleic acid library molecule. The end sequences of the linear nucleic acid library molecule comprise first and second universal adaptor sequences, respectively. In some embodiments, the first and second universal adaptor sequences of the linear library molecule comprise

binding sequences for immobilized first and second capture primers on a support, respectively.

**[0187]** The first region of the first splint strand (320) comprises a first universal adaptor sequence which can hybridize to a first universal binding sequence at one end of a linear nucleic acid library molecule (e.g., see FIGS. 1-7). The second region of the first splint strand (330) comprises a second universal adaptor sequence which can hybridize to a second universal binding sequence at the other end of the linear nucleic acid library molecule (e.g., see FIGS. 1-7). In some embodiments, the first region of the first splint strand (320) includes a first universal adaptor sequence which comprises a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer, or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the second region of the first splint strand (330) includes a second universal adaptor sequence which comprises a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer, or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the 5' end of the first splint strand (300) is phosphorylated or non-phosphorylated. In some embodiments, the 3' end of the first splint strand (300) comprises a terminal 3' OH group or a terminal 3' blocking group.

**[0188]** In some embodiments, the second splint strand (400) comprises at least two sub-regions, including a first and second sub-region (e.g., see FIGS. 2 and 3). In some embodiments, the first sub-region comprises a universal binding sequence for a third or fourth surface primer, and the second sub-region comprises a universal binding sequence for a fourth or third surface primer, wherein the first and second sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the first sub-region comprises a universal binding sequence for a third surface primer, and the second sub-region comprises a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the second splint strand (400) further comprises an optional third sub-region which includes a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN) (e.g., see FIG. 3). In some embodiments, the second splint strand (400) comprises only one sub-region and lacks a second and third sub-region, where the first sub-region comprises a sample index sequence having 5-20 bases. In some embodiments, the

sample index sequence can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay. In some embodiments, the unique identification sequence comprises a random sequence. The unique identification sequence can be designed to exhibit reduced or no hybridization to the first, second, third and fourth surface primers. An exemplary arrangement of the sub-regions in the second splint strand (400), in a 5' to 3' orientation comprises: 5' - [second sub-region] - [first sub-region] - 3'. Another exemplary arrangement of the sub-regions in the second splint strand (400), in a 5' to 3' orientation comprises: 5' - [third sub-region] - [second sub-region] - [first sub-region] - 3' (e.g., see FIGS. 2 and 3). In some embodiments, the second splint strand (400) can be 20-100 nucleotides in length, or 30-80 nucleotides in length, or 40-60 nucleotides in length. In some embodiments, the 5' end of the second splint strand (400) is phosphorylated or non-phosphorylated. In some embodiments, the 3' end of the second splint strand (400) comprises a terminal 3' OH group or a terminal 3' blocking group. In some embodiments, the second splint strands (400) comprise one or more phosphorothioate linkages at the 5' and/or 3' ends to confer exonuclease resistance. In some embodiments, the second splint strands (400) comprise one or more phosphorothioate linkages at an internal position to confer endonuclease resistance. In some embodiments, the second splint strands (400) comprise one or more 2'-O-methylcytosine bases at the 5' and/or 3' end, or at an internal position.

**[0189]** In some embodiments, the first splint strand (300) includes an internal region (310) which comprises at least two sub-regions, including a fourth and fifth sub-region (e.g., see FIGS. 2 and 3). The fourth sub-region hybridizes to the first sub-region of the second splint strand (400). The fifth sub-region hybridizes to the second sub-region of the second splint strand (400). The fourth and fifth sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the internal region (310) of the first splint strand further comprises an optional sixth sub-region which hybridizes to the third sub-region of the second splint strand (400) (e.g., see FIG. 3). An exemplary arrangement of the sub-regions of the first splint strand (300), in a 5' to 3' orientation comprises: 5' - [fourth sub-region] - [fifth sub-region] - 3'. Another exemplary arrangement of the sub-regions of the first splint strand (300), in a 5' to 3' orientation comprises: 5' - [fourth sub-region] - [fifth sub-region] - [sixth sub-region] - 3' (e.g., see FIGS. 2 and 3). In some embodiments, the first splint strand (300) can be 50-150 nucleotides in length, or 60-100 nucleotides in length, or 70-90 nucleotides in length. In some embodiments, the first splint strands (300) comprise one or more phosphorothioate linkage at the 5' and/or 3' ends to confer exonuclease resistance. In some embodiments, the first splint

strands (300) comprise one or more phosphorothioate linkage at an internal position to confer endonuclease resistance. In some embodiments, the first splint strands (300) comprise one or more 2'-O-methylcytosine bases at the 5' and/or 3' end, or at an internal position.

**[0190]** In some embodiments, the first sub-region of the second splint strand (400) comprises the sequence 5'-CATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:200). In some embodiments, the second sub-region of the second splint strand (400) comprises the sequence

5'-AGTCGTCGCAGCCTCACCTGATC-3' (SEQ ID NO:201). In some embodiments, the second splint strand (400) comprises a first and second sub-region comprising the sequence 5'-AGTCGTCGCAGCCTCACCTGATCCATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:202). See FIG. 10. In some embodiments, the 5' end of the second splint strand (400) can be phosphorylated or non-phosphorylated.

**[0191]** In some embodiments, the first region of the first splint strand (320) includes a first universal adaptor sequence which comprises a universal binding sequence (or a complementary sequence thereof) for a first surface primer, where the first region (320) comprises the sequence 5'-TCGGTGGTCGCCGTATCATT-3' (SEQ ID NO:193). For example, the first region of the first splint strand (320) can hybridize to a P5 surface primer or a complementary sequence of the P5 surface primer. For example, the P5 surface primer comprises the sequence 5'- AATGATACGGCGACCACCGA-3' (SEQ ID NO: 203; short P5), or the P5 surface primer comprises the sequence 5'-

AATGATACGGCGACCACCGAGATC-3' (SEQ ID NO:194; long P5). In some embodiments, the second region of the first splint strand (330) includes a second universal adaptor sequence which comprises a universal binding sequence (or a complementary sequence thereof) for a second surface primer, where the second region (330) comprises the sequence 5'-CAAGCAGAAGACGGCATACGA-3' (SEQ ID NO:195). For example, the second region of the first splint strand (330) can hybridize to a P7 surface primer or a complementary sequence of the P7 surface primer. For example, the P7 surface primer comprises the sequence 5'- CAAGCAGAAGACGGCATACGA-3' (SEQ ID NO:195; short P7), or the P7 surface primer comprises the sequence 5'-

CAAGCAGAAGACGGCATACGAGAT-3' (SEQ ID NO:196; long P7). In some embodiments, the first splint strand (300) includes an internal region (310) which comprises a fourth sub-region having the sequence

5'-ACCCTGAAAGTACGTGCATTACATG-3' (SEQ ID NO:197). In some embodiments, the first splint strand (300) includes an internal region (310) which comprises a fifth sub-

region having the sequence 5'-GATCAGGTGAGGCTGCGACGACT-3' (SEQ ID NO:198). In some embodiments, the first splint strand (300) comprises a first region (320), an internal region (310) having a fourth and fifth sub-region, and a second region (330), having the sequence 5'-

TCGGTGGTCGCCGTATCATTACCCTGAAAGTACGTGCATTACATGGATCAGGTGAGGCTGCGACGACTCAAGCAGAAGACGGCATAACGA-3' (SEQ ID NO:199). See FIG.

10. In some embodiments, the 5' end of the first splint strand (300) can be phosphorylated or non-phosphorylated. In some embodiments, the first sub-region of the second splint strand (400) can hybridize to the fourth sub-region of the first splint strand (300). In some embodiments, the second sub-region of the second splint strand (400) can hybridize to the fifth sub-region of the first splint strand (300).

### **Library-Splint Complexes**

**[0192]** The present disclosure provides a library-splint complex (500) 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 double-stranded splint adaptor (200) which includes a first splint strand (long splint strand (300)) and a second splint strand (short splint strand (400)), wherein the first splint strand comprises a first region (320), an internal region (310), and a second region (330), wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400) to form the double-stranded splint adaptor (200) having a double-stranded region and two flanking single-stranded regions. In the library-splint complex (500), the first region of the first splint strand (320) is hybridized to the at least first left universal adaptor sequence (120) of the library molecule, and a second region of the first splint strand (330) is hybridized to the at least first right universal sequence (130) of the library molecule, thereby circularizing the library molecule to generate a library-splint complex (500) (e.g., see FIGS. 1-7).

**[0193]** In the library-splint complex (500), the first region of the first splint strand (320) comprises a first universal adaptor sequence which can hybridize to a first universal binding sequence at one end of a linear nucleic acid library molecule (e.g., see FIGS. 1-7). In some embodiments, the first region of the first splint strand (320) includes a first universal adaptor sequence which comprises a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer, or a universal binding sequence for a

compaction oligonucleotide. In some embodiments, the first splint strand (300) can be 50-150 nucleotides in length, or 60-100 nucleotides in length, or 70-90 nucleotides in length. In some embodiments, the first splint strands (300) comprise one or more phosphorothioate linkages at the 5' and/or 3' ends to confer exonuclease resistance. In some embodiments, the first splint strands (300) comprise one or more phosphorothioate linkages at an internal position to confer endonuclease resistance. In some embodiments, the first splint strands (300) comprise one or more 2'-O-methylcytosine bases at the 5' and/or 3' end, or at an internal position. In some embodiments, the 5' end of the first splint strand (300) is phosphorylated or lacks a phosphate group. In some embodiments, the 3' end of the first splint strand (300) includes a terminal 3' OH group or a terminal 3' blocking group.

**[0194]** The second region of the first splint strand (330) comprises a second universal adaptor sequence which can hybridize to a second universal binding sequence at the other end of the linear nucleic acid library molecule (e.g., see FIGS. 1-7). In some embodiments, the second region of the first splint strand (330) includes a second universal adaptor sequence which comprises a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer, or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the 5' end of the second splint strand (400) is phosphorylated or lacks a phosphate group. In some embodiments, the 3' end of the second splint strand (400) includes a terminal 3' OH group or a terminal 3' blocking group.

**[0195]** In the library-splint complex (500), the first region of the first splint strand (320) is hybridized to the at least first left universal adaptor sequence (120) of the library molecule, and a second region of the first splint strand (330) is hybridized to the at least first right universal sequence (130) of the library molecule, thereby circularizing the library molecule to generate a library-splint complex (500). The library-splint complex (500) comprises a first nick between the 5' end of the library molecule and the 3' end of the second splint strand. The library-splint complex (500) also comprises a second nick between the 5' end of the second splint strand and the 3' end of the library molecule (e.g., see FIGS. 1-7). In some embodiments, the first and second nicks are enzymatically ligatable.

**[0196]** In the library-splint complex (500), the first region of the first splint strand (320) can hybridize to a sense or anti-sense strand of a double-stranded nucleic acid library molecule. In the library-splint complex (500), the second region of the first splint strand (330) can hybridize to a sense or anti-sense strand of a double-stranded nucleic acid library molecule.

The double-stranded nucleic acid library molecule can be denatured to generate the single-stranded sense and anti-sense library strands.

**[0197]** In the library-splint complex (500), the second splint strand (400) does not hybridize to the sequence of interest (110), and the internal region of the first splint strand (310) does not hybridize to the sequence of interest (110).

**[0198]** In the library-splint complex (500), the first region of the first splint strand (320) does not hybridize to the sequence of interest (110), and the second region of the first splint strand (330) does not hybridize to the sequence of interest (110).

**[0199]** In some embodiments, in the library-splint complex (500), the 5' end of the single-stranded library molecule (100) is phosphorylated or lacks a phosphate group. In some embodiments, the 3' end of the single-stranded library molecule includes a terminal 3' OH group or a terminal 3' blocking group.

**[0200]** 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). Exemplary library molecules (100) are shown in FIGS. 4-7. In some embodiments, the nucleic acid library molecule (100) can further comprise additional left and/or right universal adaptor sequences.

**[0201]** In some embodiments, the nucleic acid library molecule (100) further comprises a first left index sequence (160). In some embodiments, the nucleic acid library molecule (100) further comprises a first right index sequence (170). In some embodiments, the first left index sequence (160) comprises a sample index sequence. In some embodiments, the first right index sequence (170) comprises another sample index sequence. In some embodiments, the nucleic acid library molecule (100) includes a first left index sequence (160) and/or a first right index sequence (170). The sample index sequences can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay. Exemplary library molecules (100) are shown in FIGS. 4-7. A list of exemplary first left index sequences (160) and first right index sequences (170) is provided in Table 1 at FIGS. 23A-23F. The first left index sequence (160) can include a random sequence (e.g., NNN) or lack a random sequence. The first right index sequence (170) can include a random sequence (e.g., NNN) or lack a random sequence.

**[0202]** In some embodiments, the nucleic acid library molecule (100) further comprises at least one junction adaptor sequence located between any of the universal adaptor sequences described herein (e.g., see FIG. 7). For example, a first left junction adaptor sequence (125)

can be located between the first left universal adaptor sequence (120) and the first left index sequence (160). A second left junction adaptor sequence (165) can be located between the first left index sequence (160) and the second left universal adaptor sequence (140). A third junction adaptor sequence (145) can be located between the second left universal adaptor sequence (140) and the sequence-of-interest (110). A first right junction adaptor sequence (135) can be located between the first right universal sequence (130) and the first right index sequence (170). A second right junction adaptor sequence (175) can be located between the first right index sequence (170) and the second right universal adaptor sequence (150). A third right junction adaptor sequence (155) can be located between the second right universal adaptor sequence (150) and the sequence-of-interest (110). In some embodiments, the nucleic acid library molecule (100) further comprises at least one and up to ten appended universal adaptor sequences located 5' (upstream) to the first left universal adaptor sequence (120) (e.g., see FIG. 7). In some embodiments, the nucleic acid library molecule (100) further comprises at least one and up to ten appended universal adaptor sequences located 3' (downstream) to the first right universal sequence (130) (e.g., see FIG. 7). Any of the junction adaptor sequences comprise any sequence and can be 3-60 nucleotides in length and/or appended universal adaptor sequences. Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a universal sequence or a unique sequence. Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a binding sequence for an amplification primer, a sequencing primer or a compaction oligonucleotide. Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a binding sequence for an immobilized surface primer (e.g., capture primer). Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a sample index sequence. Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a unique identification sequence. Any of the junction adaptor sequences and/or appended universal adaptor sequences, particularly junction adaptor sequence (145) comprises a Tn5 transposon-end sequence 5'-AGATGTGTATAAGAGACAG-3' (SEQ ID NO:211). Any of the junction adaptor sequences and/or appended universal adaptor sequences, particularly junction adaptor sequence (155) comprises a Tn5 transposon-end sequence 5'-CTGTCTCTTATACACATCT-3' (SEQ ID NO:212). The Tn5 transposon-end sequences can be introduced into the library molecule (100) via a transposase-mediated reaction which includes contacting double-stranded input DNA (e.g., genomic DNA) with a Tn-5 type transposase enzyme, and a double-stranded oligonucleotide comprising the Tn transposon-end sequence (SEQ ID

NO:211) linked to a universal adaptor sequence or a sample index sequence under a condition that is suitable to form a transposon synaptic complex. In the double-stranded oligonucleotide, the Tn transposon-end sequence (SEQ ID NO:211) can be located 5' or 3' relative to the universal adaptor sequence or a sample index sequence.

**[0203]** Multiplex workflows are enabled by preparing sample-indexed libraries using one or both index sequences (e.g., left and/or right index sequences). The first left index sequences (160) and/or first right index sequences (170) can be employed to prepare separate sample-indexed libraries using input nucleic acids isolated from different sources. The sample-indexed libraries can be pooled together to generate a multiplex library mixture, and the pooled libraries can be amplified and/or sequenced. The sequences of the insert region along with the first left index sequence (160) and/or first right index sequence (170) can be used to identify the source of the input nucleic acids. In some embodiments, any number of sample-indexed libraries can be pooled together, for example 2-10, or 10-50, or 50-100, or 100-200, or more than 200 sample-indexed libraries can be pooled. Exemplary nucleic acid sources include naturally-occurring, recombinant, or chemically-synthesized sources. Exemplary nucleic acid sources include single cells, a plurality of cells, tissue, biological fluid, environmental sample or whole organism. Exemplary nucleic acid sources include fresh, frozen, fresh-frozen or archived sources (e.g., formalin-fixed paraffin-embedded; FFPE). The skilled artisan will recognize that the nucleic acids can be isolated from many other sources. The nucleic acid library molecules can be prepared in single-stranded or double-stranded form.

**[0204]** In some embodiments, the nucleic acid library molecule (100) further comprises: an optional first left unique identification sequence (180). In some embodiments, the nucleic acid library molecule (100) further comprises an optional first right unique identification sequence (190). In some embodiments, the first left unique identification sequence (180) and the first right unique identification sequence (190) each comprise a sequence that is used to uniquely identify an individual sequence of interest (e.g., insert sequence) to which the unique adaptors are appended in a population of other sequence of interest molecules. In some embodiments, the first left unique identification sequence (180) and/or the first right unique identification sequence (190) can be used for molecular tagging. Exemplary library molecules (100) are shown in FIGS. 4-7.

**[0205]** In some embodiments, the nucleic acid library molecule (100) comprises any one or any combination of two or more: a first left universal adaptor sequence (120); a second left universal adaptor sequence (140); a first left index sequence (160); a first left unique

identification sequence (180); a first right universal adaptor sequence (130); a second right universal adaptor sequence (150); a first right index sequence (170); and/or a first right unique identification sequence (190). Exemplary library molecules (100) are shown in FIGS. 4-7.

**[0206]** In some embodiments, the first left universal adaptor sequence (120) and/or the second left universal adaptor sequence (140), comprises: a universal binding sequence for a forward or reverse sequencing primer; a universal binding sequence for a first or second surface primer; a universal binding sequence for a forward or reverse amplification primer; and/or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the nucleic acid library molecule (100) can further comprise additional left universal adaptor sequences.

**[0207]** In some embodiments, the first right universal adaptor sequence (130) and/or the second right universal adaptor sequence (150), comprises: a universal binding sequence for a forward or reverse sequencing primer; a universal binding sequence for a first or second surface primer; a universal binding sequence for a forward or reverse amplification primer; and/or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the nucleic acid library molecule (100) can further comprise additional right universal adaptor sequences.

**[0208]** In some embodiments, the second splint strand (400) comprises at least two sub-regions, including a first and second sub-region (e.g., see FIGS. 2 and 3). The first sub-region comprises a universal binding sequence for a third surface primer, and the second sub-region comprises a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize to (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the second splint strand (400) further comprises an optional third sub-region which includes a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN) (e.g., see FIGS. 2 and 3). In some embodiments, the second splint strand (400) comprises only one sub-region and lacks a second and third sub-region, where the first sub-region comprises a sample index sequence having 5-20 bases. In some embodiments, the sample index sequence can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay. In some embodiments, the unique identification sequence comprises a random sequence. The unique identification sequence can be designed to exhibit reduced or no hybridization to the first, second, third and fourth surface primers. An exemplary arrangement of the sub-regions in the second splint strand (400), in a 5' to 3' orientation

comprises: 5' - [second sub-region] - [first sub-region] - 3'. Another exemplary arrangement of the sub-regions in the second splint strand (400), in a 5' to 3' orientation comprises: 5' - [third sub-region] - [second sub-region] - [first sub-region] - 3'. In some embodiments, the second splint strand (400) can be 20-100 nucleotides in length, or 30-80 nucleotides in length, or 40-60 nucleotides in length. In some embodiments, the second splint strands (400) comprise one or more phosphorothioate linkages at the 5' and/or 3' ends to confer exonuclease resistance. In some embodiments, the second splint strands (400) comprise one or more phosphorothioate linkages at an internal position to confer endonuclease resistance. In some embodiments, the second splint strands (400) comprise one or more 2'-O-methylcytosine bases at the 5' and/or 3' end, or at an internal position. In some embodiments, the 5' end of the second splint strand (400) is phosphorylated or non-phosphorylated. In some embodiments, the 3' end of the second splint strand (400) comprises a terminal 3' OH group or a terminal 3' blocking group.

**[0209]** In some embodiments, the first splint strand (300) includes an internal region (310) which comprises at least two sub-regions, including a fourth and fifth sub-region (e.g., see FIGS. 2 and 3). The fourth sub-region hybridizes to the first sub-region of the second splint strand (400). The fifth sub-region hybridizes to the second sub-region of the second splint strand (400). The fourth and fifth sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the internal region (310) of the first splint strand further comprises an optional sixth sub-region which hybridizes to the third sub-region of the second splint strand (400) (e.g., see FIGS. 2 and 3). An exemplary arrangement of the sub-regions of the first splint strand (300), in a 5' to 3' orientation comprises: 5' - [fourth sub-region] - [fifth sub-region] - 3'. Another exemplary arrangement of the sub-regions of the first splint strand (300), in a 5' to 3' orientation comprises: 5' - [fourth sub-region] - [fifth sub-region] - [sixth sub-region] - 3'.

**[0210]** In some embodiments, an exemplary library-splint complex (500) comprises: **(a)** single-stranded nucleic acid library molecule (100); **(b)** a first splint strand (300); and **(c)** a second splint strand (400).

**[0211]** In the exemplary library-splint complex (500), the single-stranded nucleic acid library molecule (100) comprises 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 (120); **(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 (130).

**[0212]** In the exemplary library-splint complex (500), the first splint strand (300) comprises components arranged in a 5' to 3' order: a first region (320); an internal region (310); and a second region (330).

**[0213]** In the exemplary library-splint complex (500), the second splint strand (400) comprises sub-regions arranged in a 3' to 5' order: a first sub-region having a universal binding sequence for a third surface primer; and a second sub-region having a universal binding sequence for a fourth surface primer.

**[0214]** In the exemplary library-splint complex (500), portions of the first splint strand (300) are hybridized to portions of the library molecule (100) thereby circularizing the library molecule to generate a library-splint complex (500), such that the first region (320) of the first splint strand is hybridized to the binding sequence for the first surface primer (120), and the third region (330) of the first splint strand is hybridized to the binding sequence for the second surface primer (130). Additionally, the second splint strand (400) is hybridized to the internal region (310) of the first splint strand (300). The library-splint complex (500) comprises a first nick between the 5' end of the library molecule and the 3' end of the second splint strand, and a second nick between the 5' end of the second splint strand and the 3' end of the library molecule, and the first and second nicks are enzymatically ligatable.

**[0215]** In the exemplary library-splint complex (500), the second splint strand (400) does not hybridize to the sequence of interest (110), and the internal region of the first splint strand (310) does not hybridize to the sequence of interest (110).

**[0216]** In the exemplary library-splint complex (500), the first region of the first splint strand (320) does not hybridize to the sequence of interest (110), and the second region of the first splint strand (330) does not hybridize to the sequence of interest (110).

**[0217]** In some embodiments, any of the library-splint complexes (500) describe herein comprise a plurality of library-splint complexes (500), wherein the sequence of interest (110) of individual library-splint complexes in the plurality comprise the same sequence of interest or different sequences of interest.

**[0218]** In some embodiments, in any of the library-splint complexes (500) describe herein, the first sub-region of the second splint strand (400) comprises the sequence 5'-CATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:200). In some embodiments, the second sub-region of the second splint strand (400) comprises the sequence

5'-AGTCGTCGCAGCCTCACCTGATC-3' (SEQ ID NO:201). In some embodiments, the second splint strand (400) comprises a first and second sub-region comprising the sequence 5'-AGTCGTCGCAGCCTCACCTGATCCATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:202). See FIG. 10. In some embodiments, the 5' end of the second splint strand (400) can be phosphorylated or non-phosphorylated.

In some embodiments, the second splint strand (400) comprises only one sub-region and lacks a second and third sub-region, where the first sub-region comprises a sample index sequence having 5-20 bases.

**[0219]** In some embodiments, in any of the library-splint complexes (500) describe herein, the first region of the first splint strand (320) includes a first universal adaptor sequence which comprises a universal binding sequence (or a complementary sequence thereof) for a first surface primer, where the first region (320) comprises the sequence 5'-TCGGTGGTCGCCGTATCATT-3' (SEQ ID NO:193). For example, the first region of the first splint strand (320) can hybridize to a P5 surface primer or a complementary sequence of the P5 surface primer. For example, the P5 surface primer comprises the sequence 5'-AATGATACGGCGACCACCGA-3' (SEQ ID NO: 203; short P5), or the P5 surface primer comprises the sequence

5'- AATGATACGGCGACCACCGAGATC-3' (SEQ ID NO:194; long P5). In some embodiments, the second region of the first splint strand (330) includes a second universal adaptor sequence which comprises a universal binding sequence (or a complementary sequence thereof) for a second surface primer, where the second region (330) comprises the sequence

5'-CAAGCAGAAGACGGCATAACGA-3' (SEQ ID NO:195). For example, the second region of the first splint strand (330) can hybridize to a P7 surface primer or a complementary sequence of the P7 surface primer. For example, the P7 surface primer comprises the sequence

5'- CAAGCAGAAGACGGCATAACGA-3' (SEQ ID NO:195; short P7), or the P7 surface primer comprises the sequence 5'- CAAGCAGAAGACGGCATAACGAGAT-3' (SEQ ID NO:196; long P7). In some embodiments, the first splint strand (300) includes an internal region (310) which comprises a fourth sub-region having the sequence

5'-ACCCTGAAAGTACGTGCATTACATG-3' (SEQ ID NO:197). In some embodiments, the first splint strand (300) includes an internal region (310) which comprises a fifth sub-region having the sequence 5'-GATCAGGTGAGGCTGCGACGACT'3' (SEQ ID NO:198).

In some embodiments, the first splint strand (300) comprises a first region (320), an internal

region (310) having a fourth and fifth sub-region, and a second region (330), having the sequence 5'-

TCGGTGGTCGCCGTATCATTACCCTGAAAGTACGTGCATTACATGGATCAGGTGAGGCTGCGACGACTCAAGCAGAAGACGGCATACGA-3' (SEQ ID NO:199). See FIG.

10. In some embodiments, the 5' end of the first splint strand (300) can be phosphorylated or non-phosphorylated. In some embodiments, the first sub-region of the second splint strand (400) can hybridize to the fourth sub-region of the first splint strand (300). In some embodiments, the second sub-region of the second splint strand (400) can hybridize to the fifth sub-region of the first splint strand (300).

**[0220]** In some embodiments, in any of the library-splint complexes (500) describe herein, the first region of the first splint strand (320) comprises a sequence that can bind a first left universal adaptor sequence (120) of a library molecules, where the first region of the first splint strand (320) comprises the sequence 5'- ACCCTGAAAGTACGTGCATTACATG -3' (SEQ ID NO:197) or a complementary sequence thereof.

**[0221]** In some embodiments, in any of the library-splint complexes (500) describe herein, the second region of the first splint strand (330) comprises a sequence that can bind a first right universal adaptor sequence (130) of a library molecules, where the second region of the first splint strand (330) comprises the sequence 5'- GATCAGGTGAGGCTGCGACGACT - 3' (SEQ ID NO:198) or a complementary sequence thereof.

**[0222]** In some embodiments, in any of the library-splint complexes (500) describe herein, the library molecule includes a left universal binding sequence (120) which binds the first region of the first splint strand (320), where the left universal binding sequence (120) comprises the sequence 5'- AATGATACGGCGACCACCGA-3' (SEQ ID NO:203).

**[0223]** In some embodiments, in any of the library-splint complexes (500) describe herein, the library molecule includes a left universal binding sequence (120) which binds the first region of the first splint strand (320), where the left universal binding sequence (120) comprises the sequence 5'- CATGTAATGCACGTACTTTCAGGGT -3' (SEQ ID NO:200) or a complementary sequence thereof.

**[0224]** In some embodiments, in any of the library-splint complexes (500) describe herein, the library molecule includes a left universal binding sequence for a sequencing primer (140) where the left universal binding sequence comprises the sequence 5'- ACACTCTTCCCTACACGACGCTCTCCGATCT -3' (SEQ ID NO:204).

**[0225]** In some embodiments, in any of the library-splint complexes (500) describe herein, the library molecule includes a left universal binding sequence for a sequencing primer (140) where the left universal binding sequence comprises the sequence

5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG -3' (SEQ ID NO:207).

**[0226]** In some embodiments, in any of the library-splint complexes (500) describe herein, the library molecule includes a left universal binding sequence for a sequencing primer (140) where the left universal binding sequence comprises the sequence

5'- CGTGCTGGATTGGCTCACCAGACACCTTCCGACAT -3' (SEQ ID NO:208).

**[0227]** In some embodiments, in any of the library-splint complexes (500) describe herein, the library molecule includes a left universal binding sequence for a sequencing primer (150) where the left universal binding sequence comprises the sequence

5'- AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC -3' (SEQ ID NO:205).

**[0228]** In some embodiments, in any of the library-splint complexes (500) describe herein, the library molecule includes a left universal binding sequence for a sequencing primer (150) where the left universal binding sequence comprises the sequence

5'- CTGTCTCTTATACACATCTCCGAGCCACGAGAC -3' (SEQ ID NO:209).

**[0229]** In some embodiments, in any of the library-splint complexes (500) describe herein, the library molecule includes a left universal binding sequence for a sequencing primer (150) where the left universal binding sequence comprises the sequence

5'- ATGTCGGAAGGTGTGCAGGCTACCGCTTGCAACT -3' (SEQ ID NO:210).

**[0230]** In some embodiments, in any of the library-splint complexes (500) describe herein, the library molecule includes a right universal binding sequence (130) which binds the first region of the first splint strand (330), where the right universal binding sequence (130) comprises the sequence 5'- TCGTATGCCGTCTTCTGCTTG -3' (SEQ ID NO:206).

**[0231]** In some embodiments, in any of the library-splint complexes (500) describe herein, the library molecule includes a right universal binding sequence (130) which binds the first region of the first splint strand (330), where the right universal binding sequence (130) comprises the sequence 5'- AGTCGTCGCAGCCTCACCTGATC -3' (SEQ ID NO:201) or a complementary sequence thereof.

**[0232]** The present disclosure provides a reaction mixture comprising a plurality of any of the library-splint complexes (500) described herein. In some embodiments, the reaction mixture comprises a plurality of any of the library-splint complexes (500) described herein, and a T4 polynucleotide kinase. In some embodiments, the reaction mixture comprises a plurality of any of the library-splint complexes (500) described herein, and a ligase enzyme.

In some embodiments, the reaction mixture comprises a plurality of any of the library-splint complexes (500) described herein, and a T4 polynucleotide kinase and a ligase enzyme. In some embodiments, the ligase enzyme comprises T7 DNA ligase, T3 ligase, T4 ligase, or Taq ligase.

### **Covalently Closed Circular Molecules**

**[0233]** The present disclosure provides a covalently closed circular library molecule (600) comprising: a sequence of interest (110), at least a first left universal adaptor sequence (120), at least a first right universal adaptor sequence (130), and a second splint strand sequence (400). Exemplary covalently closed circular library molecules are shown in FIG. 8. In some embodiments, the covalently closed circular library molecule (600) further comprises a second left universal adaptor sequence (140). In some embodiments, the covalently closed circular molecule (600) further comprises a second right universal adaptor sequence (150). In some embodiments, the covalently closed circular molecule (600) further comprise additional left and/or right universal adaptor sequences.

**[0234]** In some embodiments, the covalently closed circular molecule (600) further comprises a first left index sequence (160) and/or a first right index sequence (170). The sample index sequences can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay. A list of exemplary first left index sequences (160) and first right index sequences (170) is provided in Table 1 at FIGS. 23A-23F. The first left index sequence (160) can include a random sequence (e.g., NNN) or lack a random sequence. The first right index sequence (170) can include a random sequence (e.g., NNN) or lack a random sequence.

**[0235]** Multiplex workflows are enabled by preparing sample-indexed libraries using one or both index sequences (e.g., left and/or right index sequences). The first left index sequences (160) and/or first right index sequences (170) can be employed to prepare separate sample-indexed libraries using input nucleic acids isolated from different sources. The sample-indexed libraries can be pooled together to generate a multiplex library mixture, and the pooled libraries can be amplified and/or sequenced. The sequences of the insert region along with the first left index sequence (160) and/or first right index sequence (170) can be used to identify the source of the input nucleic acids. In some embodiments, any number of sample-indexed libraries can be pooled together, for example 2-10, or 10-50, or 50-100, or 100-200, or more than 200 sample-indexed libraries can be pooled. Exemplary nucleic acid sources include naturally-occurring, recombinant, or chemically-synthesized sources. Exemplary

nucleic acid sources include single cells, a plurality of cells, tissue, biological fluid, environmental sample or whole organism. Exemplary nucleic acid sources include fresh, frozen, fresh-frozen or archived sources (e.g., formalin-fixed paraffin-embedded; FFPE). The skilled artisan will recognize that the nucleic acids can be isolated from many other sources. The nucleic acid library molecules can be prepared in single-stranded or double-stranded form.

**[0236]** In some embodiments, the covalently closed circular molecule (600) further comprises an optional first left unique identification sequence (180) and/or an optional first right unique identification sequence (190). Although FIG. 8 shows an exemplary covalently closed circular molecule (600) having a first left unique identification sequence (180), the skilled artisan will recognize that a linear library molecule (100) such as the one shown in FIG. 6 can be circularized using a double stranded splint adaptor (200) to generate a covalently closed circular molecule (600) having a first right unique identification sequence (190). In some embodiments, the first left unique identification sequence (180) and the first right unique identification sequence (190) each comprise a sequence that is used to uniquely identify an individual sequence of interest (e.g., insert sequence) to which the unique adaptors are appended in a population of other sequence of interest molecules. In some embodiments, the first left unique identification sequence (180) and/or the first right unique identification sequence (190) can be used for molecular tagging.

**[0237]** In some embodiments, the covalently closed circular molecule (600) comprises any one or any combination of two or more: a first left universal adaptor sequence (120); a second left universal adaptor sequence (140); a first left index sequence (160); a first left unique identification sequence (180); a first right universal adaptor sequence (130); a second right universal adaptor sequence (150); a first right index sequence (170); and/or a first right unique identification sequence (190). In some embodiments, the first left index sequence (160) comprises a sample index sequence. In some embodiments, the first right index sequence (170) comprises another sample index sequence. The sample index sequences can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay. In some embodiments, the first left unique identification sequence (180) and the first right unique identification sequence (190) each comprise a sequence that is used to uniquely identify an individual sequence of interest (e.g., insert sequence) to which the unique adaptors are appended in a population of other sequence of interest molecules. In some embodiments, the first left unique identification sequence (180) and/or the first right unique identification sequence (190) can be used for molecular tagging.

**[0238]** In some embodiments, in the covalently closed circular molecule (600), the first left universal adaptor sequence (120) and/or the second left universal adaptor sequence (140), comprises: a universal binding sequence for a forward or reverse sequencing primer; a universal binding sequence for a first or second surface primer; a universal binding sequence for a forward or reverse amplification primer; and/or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the covalently closed circular molecule (600) can further comprise additional left universal adaptor sequences.

**[0239]** In some embodiments, in the covalently closed circular molecule (600), the first right universal adaptor sequence (130) and/or the second right universal adaptor sequence (150), comprises: a universal binding sequence for a forward or reverse sequencing primer; a universal binding sequence for a first or second surface primer; a universal binding sequence for a forward or reverse amplification primer; and/or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the covalently closed circular molecule (600) can further comprise additional right universal adaptor sequences.

**[0240]** In some embodiments, the covalently closed circular molecule (600) further comprises at least one junction adaptor sequence located between any of the universal adaptor sequences described herein (e.g., see FIG. 7). For example, a first left junction adaptor sequence (125) can be located between the first left universal adaptor sequence (120) and the first left index sequence (160). A second left junction adaptor sequence (165) can be located between the first left index sequence (160) and the second left universal adaptor sequence (140). A third junction adaptor sequence (145) can be located between the second left universal adaptor sequence (140) and the sequence-of-interest (110). A first right junction adaptor sequence (135) can be located between the first right universal sequence (130) and the first right index sequence (170). A second right junction adaptor sequence (175) can be located between the first right index sequence (170) and the second right universal adaptor sequence (150). A third right junction adaptor sequence (155) can be located between the second right universal adaptor sequence (150) and the sequence-of-interest (110). In some embodiments, the covalently closed circular molecule (600) further comprises at least one and up to ten appended universal adaptor sequences located 5' (upstream) to the first left universal adaptor sequence (120) (e.g., see FIG. 7). In some embodiments, the covalently closed circular molecule (600) further comprises at least one and up to ten appended universal adaptor sequences located 3' (downstream) to the first right universal sequence (130) (e.g., see FIG. 7). Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise any sequence and can be 3-60 nucleotides in length. Any of the

junction adaptor sequences and/or appended universal adaptor sequences comprise a universal sequence or a unique sequence. Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a binding sequence for an amplification primer, a sequencing primer or a compaction oligonucleotide. Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a binding sequence for an immobilized surface primer (e.g., capture primer). Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a sample index sequence. Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a unique identification sequence. Any of the junction adaptor sequences and/or appended universal adaptor sequences, particularly junction adaptor sequence (145) comprises a Tn5 transposon-end sequence 5'-AGATGTGTATAAGAGACAG-3' (SEQ ID NO:211). Any of the junction adaptor sequences and/or appended universal adaptor sequences, particularly junction adaptor sequence (155) comprises a Tn5 transposon-end sequence 5'-CTGTCTCTTATACACATCT-3' (SEQ ID NO:212). The Tn5 transposon-end sequences can be introduced into the library molecule (100) via a transposase-mediated reaction which includes contacting double-stranded input DNA (e.g., genomic DNA) with a Tn-5 type transposase enzyme, and a double-stranded oligonucleotide comprising the Tn transposon-end sequence (SEQ ID NO:211) linked to a universal adaptor sequence or a sample index sequence under a condition that is suitable to form a transposon synaptic complex. In the double-stranded oligonucleotide, the Tn transposon-end sequence (SEQ ID NO:211) can be located 5' or 3' relative to the universal adaptor sequence or a sample index sequence.

**[0241]** In some embodiments, the second splint strand sequence (400) of the covalently closed circular molecule comprises at least two sub-regions, including a first and second sub-region. The first sub-region comprises a universal binding sequence for a third surface primer, and the second sub-region comprises a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize to (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the second splint strand (400) further comprises an optional third sub-region which includes a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN). In some embodiments, the second splint strand (400) comprises only one sub-region and lacks a second and third sub-region, where the first sub-region comprises a sample index sequence having 5-20 bases. In some embodiments, the sample index sequence can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay. In some embodiments, the

unique identification sequence comprises a random sequence. The unique identification sequence can be designed to exhibit reduced or no hybridization to the first, second, third and fourth surface primers. An exemplary arrangement of the sub-regions in the second splint strand (400), in a 5' to 3' orientation comprises: 5'- [second sub-region] – [first sub-region] – 3'. Another exemplary arrangement of the sub-regions in the second splint strand (400), in a 5' to 3' orientation comprises: 5'- [third sub-region] – [second sub-region] – [first sub-region] – 3'.

**[0242]** In some embodiments, the second splint strand sequence (400) of the covalently closed circular library molecule (600) can be hybridized to a first splint strand (300). In some embodiments, the first splint strand (300) includes an internal region (310) which comprises at least two sub-regions, including a fourth and fifth sub-region. The fourth sub-region hybridizes to the first sub-region of the second splint strand (400). The fifth sub-region hybridizes to the second sub-region of the second splint strand (400). The fourth and fifth sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the internal region (310) of the first splint strand further comprises an optional sixth sub-region which hybridizes to the third sub-region of the second splint strand (400). An exemplary arrangement of the sub-regions of the first splint strand (300), in a 5' to 3' orientation comprises: 5' – [fourth sub-region] – [fifth sub-region] – 3'. Another exemplary arrangement of the sub-regions of the first splint strand (300), in a 5' to 3' orientation comprises: 5' – [fourth sub-region] – [fifth sub-region] – [sixth sub-region] – 3'.

**[0243]** In some embodiments, an exemplary covalently closed circular molecule (600) comprises: **(i)** a first left universal adaptor sequence (120) having a binding sequence for a first surface primer (120); **(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; **(v)** a first right universal adaptor sequence (130) having a binding sequence for a second surface primer (130); and **(vi)** a second splint strand sequence (400), wherein the covalently closed circular molecule (600) is optionally hybridized to the first splint strand (300).

**[0244]** In the exemplary covalently closed circular molecule (600), the second splint strand region (400) comprises at least two sub-regions, including a first and second sub-region. The first sub-region comprises a universal binding sequence for a third surface primer, and the second sub-region comprises a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize to (or at least exhibit very little

hybridization to) the first and second surface primers. In some embodiments, the second splint strand (400) further comprises an optional third sub-region which includes a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN). In some embodiments, the second splint strand (400) comprises only one sub-region and lacks a second and third sub-region, where the first sub-region comprises a sample index sequence having 5-20 bases. In some embodiments, the sample index sequence can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay. In some embodiments, the unique identification sequence comprises a random sequence. The unique identification sequence can be designed to exhibit reduced or no hybridization to the first, second, third and fourth surface primers. An exemplary arrangement of the sub-regions in the second splint strand (400), in a 5' to 3' orientation comprises: 5' - [second sub-region] – [first sub-region] – 3'. Another exemplary arrangement of the sub-regions in the second splint strand (400), in a 5' to 3' orientation comprises: 5' - [third sub-region] – [second sub-region] – [first sub-region] – 3'.

**[0245]** In some embodiments, any of the covalently closed circular molecules (600) described herein comprise a plurality of covalently closed circular molecules (600), wherein the sequence of interest (110) of individual covalently closed circular molecules (600) in the plurality comprise the same sequence of interest or different sequences of interest.

**[0246]** In some embodiments, in any of the covalently closed circular molecules (600) described herein, the first sub-region of the second splint strand (400) comprises the sequence 5'-CATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:200). In some embodiments, the second sub-region of the second splint strand (400) comprises the sequence 5'-AGTCGTCGCAGCCTCACCTGATC-3' (SEQ ID NO:201). In some embodiments, the second splint strand (400) comprises a first and second sub-region comprising the sequence 5'-AGTCGTCGCAGCCTCACCTGATCCATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:202). See FIG. 10. In some embodiments, the 5' end of the second splint strand (400) can be phosphorylated or non-phosphorylated.

**[0247]** In some embodiments, in any of the covalently closed circular molecules (600) described herein, the first region of the first splint strand (320) includes a first universal adaptor sequence which comprises a universal binding sequence (or a complementary sequence thereof) for a first surface primer, where the first region (320) comprises the sequence 5'-TCGGTGGTCGCCGTATCATT-3' (SEQ ID NO:193). For example, the first region of the first splint strand (320) can hybridize to a P5 surface primer or a complementary sequence

of the P5 surface primer. For example, the P5 surface primer comprises the sequence 5'-AATGATACGGCGACCACCGA-3' (SEQ ID NO:203; short P5), or the P5 surface primer comprises the sequence 5'-AATGATACGGCGACCACCGAGATC-3' (SEQ ID NO:194; long P5). In some embodiments, the second region of the first splint strand (330) includes a second universal adaptor sequence which comprises a universal binding sequence (or a complementary sequence thereof) for a second surface primer, where the second region (330) comprises the sequence 5'-CAAGCAGAAGACGGCATACGA-3' (SEQ ID NO:195). For example, the second region of the first splint strand (330) can hybridize to a P7 surface primer or a complementary sequence of the P7 surface primer. For example, the P7 surface primer comprises the sequence 5'-CAAGCAGAAGACGGCATACGA-3' (SEQ ID NO:195; short P7), or the P7 surface primer comprises the sequence 5'-CAAGCAGAAGACGGCATACGAGAT-3' (SEQ ID NO:196; long P7). In some embodiments, the first splint strand (300) includes an internal region (310) which comprises a fourth sub-region having the sequence

5'-ACCCTGAAAGTACGTGCATTACATG-3' (SEQ ID NO:197). In some embodiments, the first splint strand (300) includes an internal region (310) which comprises a fifth sub-region having the sequence 5'-GATCAGGTGAGGCTGCGACGACT-3' (SEQ ID NO:198). In some embodiments, the first splint strand (300) comprises a first region (320), an internal region (310) having a fourth and fifth sub-region, and a second region (330), having the sequence 5'-

TCGGTGGTCGCCGTATCATTACCCTGAAAGTACGTGCATTACATGGATCAGGTGAGGCTGCGACGACTCAAGCAGAAGACGGCATACGA-3' (SEQ ID NO:199). See

FIG.10. In some embodiments, the 5' end of the first splint strand (300) can be phosphorylated or non-phosphorylated. In some embodiments, the first sub-region of the second splint strand (400) can hybridize to the fourth sub-region of the first splint strand (300). In some embodiments, the second sub-region of the second splint strand (400) can hybridize to the fifth sub-region of the first splint strand (300).

**[0248]** In some embodiments, in any of the covalently closed circular molecules (600) described herein, the first region of the first splint strand (320) comprises a sequence that can bind a first left universal adaptor sequence (120) of a library molecules, where the first region of the first splint strand (320) comprises the sequence 5'-ACCCTGAAAGTACGTGCATTACATG-3' (SEQ ID NO:197) or a complementary sequence thereof.

**[0249]** In some embodiments, in any of the covalently closed circular molecules (600) described herein, the second region of the first splint strand (330) comprises a sequence that can bind a first right universal adaptor sequence (130) of a library molecules, where the second region of the first splint strand (330) comprises the sequence 5'-GATCAGGTGAGGCTGCGACGACT -3' (SEQ ID NO:198) or a complementary sequence thereof.

**[0250]** In some embodiments, in any of the covalently closed circular molecules (600) described herein, the library molecule includes a left universal binding sequence (120) which comprises the sequence 5'-AATGATACGGCGACCACCGA-3' (SEQ ID NO:203).

**[0251]** In some embodiments, in any of the covalently closed circular molecules (600) described herein, the library molecule includes a left universal binding sequence (120) which binds the first region of the first splint strand (320), where the left universal binding sequence (120) comprises the sequence 5'-CATGTAATGCACGTACTTTCAGGGT -3' (SEQ ID NO:200) or a complementary sequence thereof.

**[0252]** In some embodiments, in any of the covalently closed circular molecules (600) described herein, the library molecule includes a left universal binding sequence for a sequencing primer (140) where the left universal binding sequence comprises the sequence 5'-ACACTCTTCCCTACACGACGCTCTTCCGATCT -3' (SEQ ID NO:204).

**[0253]** In some embodiments, in any of the covalently closed circular molecules (600) described herein, the library molecule includes a left universal binding sequence for a sequencing primer (140) where the left universal binding sequence comprises the sequence 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG -3' (SEQ ID NO:207).

**[0254]** In some embodiments, in any of the covalently closed circular molecules (600) described herein, the library molecule includes a left universal binding sequence for a sequencing primer (140) where the left universal binding sequence comprises the sequence 5'-CGTGCTGGATTGGCTCACCAGACACCTTCCGACAT -3' (SEQ ID NO:208).

**[0255]** In some embodiments, in any of the covalently closed circular molecules (600) described herein, the library molecule includes a left universal binding sequence for a sequencing primer (150) where the left universal binding sequence comprises the sequence 5'-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC -3' (SEQ ID NO:205).

**[0256]** In some embodiments, in any of the covalently closed circular molecules (600) described herein, the library molecule includes a left universal binding sequence for a sequencing primer (150) where the left universal binding sequence comprises the sequence 5'-CTGTCTCTTATACACATCTCCGAGCCCACGAGAC -3' (SEQ ID NO:209).

[0257] In some embodiments, in any of the covalently closed circular molecules (600) described herein, the library molecule includes a left universal binding sequence for a sequencing primer (150) where the left universal binding sequence comprises the sequence 5'- ATGTCGGAAGGTGTGCAGGCTACCGCTTGTCAACT -3' (SEQ ID NO:210).

[0258] In some embodiments, in any of the covalently closed circular molecules (600) described herein, the library molecule includes a right universal binding sequence (130) which comprises the sequence 5'- TCGTATGCCGTCTTCTGCTTG -3' (SEQ ID NO:206).

[0259] In some embodiments, in any of the covalently closed circular molecules (600) described herein, the library molecule includes a right universal binding sequence (130) which binds the first region of the first splint strand (330), where the right universal binding sequence (130) comprises the sequence 5'- AGTCGTCGCAGCCTCACCTGATC -3' (SEQ ID NO:201) or a complementary sequence thereof.

[0260] The present disclosure provides a reaction mixture comprising a plurality of any of the covalently closed circular molecules (600) described herein and at least one exonuclease enzyme. In some embodiments, the exonuclease enzyme comprises any one or any combination of two or more of exonuclease I, thermolabile exonuclease I and/or T7 exonuclease.

### **Kits Comprising Double-Stranded Splint Adaptors**

[0261] The present disclosure provides a kit for the use of introducing one or more new adaptor sequences into linear nucleic acid library molecules. In some embodiments, the kit can be used to circularize single-stranded nucleic acid library molecules having 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). In some embodiments, the circularized library molecules can be converted to covalently closed circular molecules which can be subjected to a rolling circle amplification (RCA) reaction to generate nucleic acid concatemers. The concatemers can be immobilized to a support for massively parallel sequencing.

[0262] The present disclosure provides kits comprising nucleic acid double-stranded splint adaptors (200), comprising: **(i)** a first splint strand (long splint strand (300)) which is hybridized to **(ii)** a second splint strand (short splint strand (400)). The first splint strand comprises a first region (320), an internal region (310), and a second region (330). The internal region of the first splint strand (310) is hybridized to the second splint strand (400) to form a double-stranded splint adaptor (200) having a double-stranded region and two

flanking single-stranded regions. The second splint strand (400) includes a new adaptor sequence that can be introduced the linear nucleic acid library molecules. Exemplary double-stranded splint adaptors are shown in FIGS. 1-7. The kit can include a container which contains the first splint strands (300) hybridized to the second splint strands (400). The kit can include a first container which contains the first splint strands (300) and a second container which contains the second splint strands (400).

**[0263]** In some embodiments, in the kit, the second splint strand (400) comprises at least two sub-regions, including a first and second sub-region (e.g., see FIGS. 2 and 3). The first sub-region comprises a universal binding sequence for a third surface primer, and the second sub-region comprises a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize to (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the second splint strand (400) further comprises an optional third sub-region which includes a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN) (e.g., see FIG. 3). In some embodiments, the second splint strand (400) comprises only one sub-region and lacks a second and third sub-region, where the first sub-region comprises a sample index sequence having 5-20 bases. In some embodiments, the sample index sequence can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay. In some embodiments, the unique identification sequence comprises a random sequence. The unique identification sequence can be designed to exhibit reduced or no hybridization to the first, second, third and fourth surface primers. An exemplary arrangement of the sub-regions in the second splint strand (400), in a 5' to 3' orientation comprises: 5' - [second sub-region] – [first sub-region] – 3'. Another exemplary arrangement of the sub-regions in the second splint strand (400), in a 5' to 3' orientation comprises: 5' - [third sub-region] – [second sub-region] – [first sub-region] – 3'. Exemplary first (300) and second (400) splint strands are shown in FIGS. 2 and 3. In some embodiments, the second splint strand (400) can be 20-100 nucleotides in length, or 30-80 nucleotides in length, or 40-60 nucleotides in length. In some embodiments, the second splint strands (400) comprise one or more phosphorothioate linkage at the 5' and/or 3' ends to confer exonuclease resistance. In some embodiments, the second splint strands (400) comprise one or more phosphorothioate linkage at an internal position to confer endonuclease resistance. In some embodiments, the second splint strands (400) comprise one or more 2'-O-methylcytosine bases at the 5' and/or 3' end, or at an internal position. In some embodiments, the 5' end of the second splint strand (400) is phosphorylated or non-phosphorylated. In some

embodiments, the 3' end of the second splint strand (400) comprises a terminal 3' OH group or a terminal 3' blocking group.

**[0264]** In some embodiments, in the kit, the first splint strand (300) comprises a first region (320), a second region (330), and internal region (310). The first region (320) comprises a first universal adaptor sequence which can hybridize to the first universal binding sequence at one end of the linear nucleic acid library molecule. The second region (330) comprises a second universal adaptor sequence which can hybridize to the second universal binding sequence at the other end of the linear nucleic acid library molecule. In some embodiments, the first region of the first splint strand (320) includes a first universal adaptor sequence which comprises a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer, or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the second region of the first splint strand (330) includes a second universal adaptor sequence which comprises a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer, or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the first splint strand (300) can be 50-150 nucleotides in length, or 60-100 nucleotides in length, or 70-90 nucleotides in length. In some embodiments, the first splint strands (300) comprise one or more phosphorothioate linkage at the 5' and/or 3' ends to confer exonuclease resistance. In some embodiments, the first splint strands (300) comprise one or more phosphorothioate linkage at an internal position to confer endonuclease resistance. In some embodiments, the first splint strands (300) comprise one or more 2'-O-methylcytosine bases at the 5' and/or 3' end, or at an internal position. In some embodiments, the 5' end of the first splint strand (300) is phosphorylated or non-phosphorylated. In some embodiments, the 3' end of the first splint strand (300) comprises a terminal 3' OH group or a terminal 3' blocking group.

**[0265]** In some embodiments, in the kit, the first splint strand (300) includes an internal region (310) which comprises at least two sub-regions, including a fourth and fifth sub-region (e.g., FIGS. 2 and 3). The fourth sub-region hybridizes to the first sub-region of the second splint strand (400). The fifth sub-region hybridizes to the second sub-region of the second splint strand (400). The fourth and fifth sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the internal region (310) of the first splint strand further comprises an optional sixth sub-region

which hybridizes to the third sub-region of the second splint strand (400). An exemplary arrangement of the sub-regions of the first splint strand (300), in a 5' to 3' orientation comprises: 5' – [fourth sub-region] – [fifth sub-region] – 3'. Another exemplary arrangement of the sub-regions of the first splint strand (300), in a 5' to 3' orientation comprises: 5' – [fourth sub-region] – [fifth sub-region] – [sixth sub-region] – 3'. Exemplary first splint strands (300) are shown in FIGS. 2 and 3.

**[0266]** In some embodiments, in any of the kits described herein, the first sub-region of the second splint strand (400) comprises the sequence 5'-

CATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:200). In some embodiments, the second sub-region of the second splint strand (400) comprises the sequence 5'-

AGTCGTCGCAGCCTCACCTGATC-3' (SEQ ID NO:201). In some embodiments, the second splint strand (400) comprises a first and second sub-region comprising the sequence 5'-AGTCGTCGCAGCCTCACCTGATCCATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:202). See FIG. 10. In some embodiments, the 5' end of the second splint strand (400) can be phosphorylated or non-phosphorylated.

**[0267]** In some embodiments, in any of the kits described herein, the first region of the first splint strand (320) includes a first universal adaptor sequence which comprises a universal binding sequence (or a complementary sequence thereof) for a first surface primer, where the first region (320) comprises the sequence 5'-TCGGTGGTCGCCGTATCATT-3' (SEQ ID NO:193). For example, the first region of the first splint strand (320) can hybridize to a P5 surface primer or a complementary sequence of the P5 surface primer. For example, the P5 surface primer comprises the sequence 5'-AATGATACGGCGACCACCGA-3' (SEQ ID NO: 203; short P5), or the P5 surface primer comprises the sequence 5'-

AATGATACGGCGACCACCGAGATC-3' (SEQ ID NO:194; long P5). In some embodiments, the second region of the first splint strand (330) includes a second universal adaptor sequence which comprises a universal binding sequence (or a complementary sequence thereof) for a second surface primer, where the second region (330) comprises the sequence

5'-CAAGCAGAAGACGGCATAACGA-3' (SEQ ID NO:195). For example, the second region of the first splint strand (330) can hybridize to a P7 surface primer or a complementary sequence of the P7 surface primer. For example, the P7 surface primer comprises the sequence 5'-CAAGCAGAAGACGGCATAACGA-3' (SEQ ID NO:195; short P7), or the P7 surface primer comprises the sequence 5'-CAAGCAGAAGACGGCATAACGAGAT-3'

(SEQ ID NO:196; long P7). In some embodiments, the first splint strand (300) includes an internal region (310) which comprises a fourth sub-region having the sequence 5'-ACCCTGAAAGTACGTGCATTACATG-3' (SEQ ID NO:197). In some embodiments, the first splint strand (300) includes an internal region (310) which comprises a fifth sub-region having the sequence 5'-GATCAGGTGAGGCTGCGACGACT-3' (SEQ ID NO:198). In some embodiments, the first splint strand (300) comprises a first region (320), an internal region (310) having a fourth and fifth sub-region, and a second region (330), having the sequence 5'-

TCGGTGGTCGCCGTATCATTACCCTGAAAGTACGTGCATTACATGGATCAGGTGAGGCTGCGACGACTCAAGCAGAAGACGGCATACTGA-3' (SEQ ID NO:199). See FIG. 10. In some embodiments, the 5' end of the first splint strand (300) can be phosphorylated or non-phosphorylated. In some embodiments, the first sub-region of the second splint strand (400) can hybridize to the fourth sub-region of the first splint strand (300). In some embodiments, the second sub-region of the second splint strand (400) can hybridize to the fifth sub-region of the first splint strand (300).

**[0268]** In some embodiments, in any of the kits described herein, the first region of the first splint strand (320) comprises a sequence that can bind a first left universal adaptor sequence (120) of a library molecules, where the first region of the first splint strand (320) comprises the sequence 5'- ACCCTGAAAGTACGTGCATTACATG -3' (SEQ ID NO:197) or a complementary sequence thereof.

**[0269]** In some embodiments, in any of the kits described herein, the second region of the first splint strand (330) comprises a sequence that can bind a first right universal adaptor sequence (130) of a library molecules, where the second region of the first splint strand (330) comprises the sequence 5'- GATCAGGTGAGGCTGCGACGACT -3' (SEQ ID NO:198) or a complementary sequence thereof.

**[0270]** In some embodiments, the kits further comprise an adaptor having a left universal binding sequence (120) which binds the first region of the first splint strand (320) for use in preparing a plurality of library molecules, where the library molecule will comprise the sequence 5'- AATGATACGGCGACCACCGA-3' (SEQ ID NO:203). The adaptor can be a single-stranded adaptor (e.g., PCR primer), double-stranded adaptor, bubble adaptor, or Y-shaped adaptor.

**[0271]** In some embodiments, in any of the kits described herein, the library molecule includes a left universal binding sequence (120) which binds the first region of the first splint strand (320), where the left universal binding sequence (120) comprises the sequence 5'-

CATGTAATGCACGTACTTTCAGGGT -3' (SEQ ID NO:200) or a complementary sequence thereof.

**[0272]** In some embodiments, the kits further comprise an adaptor having a left universal binding sequence for a sequencing primer (140) for use in preparing a plurality of library molecules, where the library molecule will comprise the sequence

5'- ACACTCTTTCCTACACGACGCTCTTCCGATCT -3' (SEQ ID NO:204). In some embodiments, the adaptor having a left universal binding sequence for a sequencing primer (140) also includes a sample index sequence (160). The adaptor can be a single-stranded adaptor (e.g., PCR primer), double-stranded adaptor, bubble adaptor, or Y-shaped adaptor.

**[0273]** In some embodiments, the kits further comprise an adaptor having a left universal binding sequence for a sequencing primer (140) for use in preparing a plurality of library molecules, where the library molecule will comprise the sequence

5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG -3' (SEQ ID NO:207). In some embodiments, the adaptor having a left universal binding sequence for a sequencing primer (140) also includes a sample index sequence (160). The adaptor can be a single-stranded adaptor (e.g., PCR primer), double-stranded adaptor, bubble adaptor, or Y-shaped adaptor.

**[0274]** In some embodiments, the kits further comprise an adaptor having a left universal binding sequence for a sequencing primer (140) for use in preparing a plurality of library molecules, where the library molecule will comprise the sequence

5'- CGTGCTGGATTGGCTCACCAGACACCTTCCGACAT -3' (SEQ ID NO:208). In some embodiments, the adaptor having a left universal binding sequence for a sequencing primer (140) also includes a sample index sequence (160). The adaptor can be a single-stranded adaptor (e.g., PCR primer), double-stranded adaptor, bubble adaptor, or Y-shaped adaptor.

**[0275]** In some embodiments, the kits further comprise an adaptor having a right universal binding sequence for a sequencing primer (150) for use in preparing a plurality of library molecules, where the library molecule will comprise the sequence

5'- AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC -3' (SEQ ID NO:205). In some embodiments, the adaptor having a right universal binding sequence for a sequencing primer (150) also includes a sample index sequence (170). The adaptor can be a single-stranded adaptor (e.g., PCR primer), double-stranded adaptor, bubble adaptor, or Y-shaped adaptor.

**[0276]** In some embodiments, the kits further comprise an adaptor having a right universal binding sequence for a sequencing primer (150) for use in preparing a plurality of library molecules, where the library molecule will comprise the sequence

5'- CTGTCTCTTATACACATCTCCGAGCCCACGAGAC -3' (SEQ ID NO:209). In some embodiments, the adaptor having a right universal binding sequence for a sequencing primer (150) also includes a sample index sequence (170). The adaptor can be a single-stranded adaptor (e.g., PCR primer), double-stranded adaptor, bubble adaptor, or Y-shaped adaptor.

**[0277]** In some embodiments, the kits further comprise an adaptor having a right universal binding sequence for a sequencing primer (150) for use in preparing a plurality of library molecules, where the library molecule will comprise the sequence

5'- ATGTCGGAAGGTGTGCAGGCTACCGCTTGTCAACT -3' (SEQ ID NO:210). In some embodiments, the adaptor having a right universal binding sequence for a sequencing primer (150) also includes a sample index sequence (170). The adaptor can be a single-stranded adaptor (e.g., PCR primer), double-stranded adaptor, bubble adaptor, or Y-shaped adaptor.

**[0278]** In some embodiments, the kits further comprise an adaptor having a right universal binding sequence (130) which binds the first region of the first splint strand (330), for use in preparing a plurality of library molecules, where the library molecule will comprise the sequence 5'- TCGTATGCCGTCTTCTGCTTG -3' (SEQ ID NO:206). The adaptor can be a single-stranded adaptor (e.g., PCR primer), double-stranded adaptor, bubble adaptor, or Y-shaped adaptor.

**[0279]** In some embodiments, in any of the kits described herein, the library molecule includes a right universal binding sequence (130) which binds the first region of the first splint strand (330), where the right universal binding sequence (130) comprises the sequence 5'- AGTCGTCGCAGCCTCACCTGATC -3' (SEQ ID NO:201) or a complementary sequence thereof.

**[0280]** In some embodiments, the kit further comprises a plurality of first left indexes (160) and/or a plurality of first right index sequence (170). In some embodiments, the kit can include separate containers holding individual first left index (160) or individual first right index (170). In some embodiments, the kit can include separate containers holding a pair of individual first left index (160) and individual first right index (170). In some embodiments, the kit contains the first left indexes (160) and/or a plurality of first right index sequence (170) in multi-well plates (e.g., 96-well plate). A list of exemplary first left index sequences (160) and first right index sequences (170) is provided in Table 1 at FIGS. 23A-23F. The first left index sequence (160) can include a random sequence (e.g., NNN) or lack a random sequence. The first right index sequence (170) can include a random or sequence (e.g., NNN) or lack a random sequence.

[0281] In some embodiments, the kit comprises nucleic acid double-stranded splint adaptors (200) and further comprises a T4 polynucleotide kinase. In some embodiments, the kit further comprises a ligase enzyme, wherein the ligase enzyme comprises T7 DNA ligase, T3 ligase, T4 ligase, or Taq ligase. In some embodiments, the kit further comprises at least one endonuclease, which comprises any one or any combination of two or more of exonuclease I, thermolabile exonuclease I and/or T7 exonuclease.

[0282] In some embodiments, the kit comprises at least one buffer for hybridizing the plurality of the double-stranded splint adaptors (200) and the plurality of nucleic acid library molecules (100). In some embodiments, the kit comprises one buffer for conducting multiple enzymatic reactions in a single reaction vessel, including any combination of (i) phosphorylating the 5' ends of the first and/or second splint strands (e.g., (300) and/or (400)), (ii) ligating the nicks in the library-splint complex (500), and/or (iii) exonuclease digestion of the first splint strand (300) from the covalently closed circular molecule (600). Alternatively, the kit comprises two or more separate buffers, where the first buffer can be used to conduct the phosphorylation reaction, the second buffer can be used to conduct the ligation reaction, and a third buffer can be used to conduct the exonuclease digestion reaction.

[0283] In some embodiments, the kit comprises one or more containers that contain any of the double-stranded splint adaptors (200) described herein, or any of the first and second splint strands (300) and (400), described herein. The kit can further comprise one or more containers that contain a T4 polynucleotide kinase, at least one ligase and/or at least one exonuclease. The kit can comprise any of these components in any combination and can be contained in a single container, or can be contained in separate container, or any combination thereof.

[0284] The kit can include instructions for use of the kit for conducting reactions to introduce one or more new adaptor sequences into linear nucleic acid library molecules.

### **Methods for Forming a Plurality of Library-Splint Complexes**

[0285] The present disclosure provides methods for forming a plurality of library-splint complexes (500) comprising: **(a)** providing a plurality of double-stranded splint adaptors (200) wherein individual double-stranded splint adaptors (200) in the plurality comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the double-stranded splint adaptor includes a double-stranded region and two flanking single-stranded regions, wherein the first splint strand comprises a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is

hybridized to the second splint strand (400). Exemplary double-stranded splint adaptors (200) are shown in FIGS. 1-7.

**[0286]** The methods for forming a plurality of library-splint complexes (500) further comprise **step (b)**: hybridizing the plurality of double-stranded splint adaptors with a plurality of single-stranded nucleic acid library molecule (100) wherein individual library molecules include 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) (e.g., FIGS. 1-7). The hybridizing is conducted under a condition suitable for hybridizing the first region of the first splint strand (320) to the at least first left universal adaptor sequence (120) of the library molecule, and the condition is suitable for hybridizing the second region of the first splint strand (330) to the at least first right universal sequence (130) of the library molecule, thereby circularizing the plurality of library molecules to form a plurality of library-splint complexes (500).

**[0287]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the first region of the first splint strand (320) comprises a first universal adaptor sequence which can hybridize to a first universal binding sequence at one end of a linear nucleic acid library molecule. In some embodiments, the first region of the first splint strand (320) includes a first universal adaptor sequence which comprises a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer, or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the 5' end of the first splint strand (300) is phosphorylated or lacks a phosphate group. In some embodiments, the 3' end of the first splint strand (300) includes a terminal 3' OH group or a terminal 3' blocking group.

**[0288]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the second region of the first splint strand (330) comprises a second universal adaptor sequence which can hybridize to a second universal binding sequence at the other end of the linear nucleic acid library molecule. In some embodiments, the second region of the first splint strand (330) includes a second universal adaptor sequence which comprises a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer, or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the 5' end of the second splint strand (400) is

phosphorylated or lacks a phosphate group. In some embodiments, the 3' end of the second splint strand (400) includes a terminal 3' OH group or a terminal 3' blocking group.

**[0289]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the first region of the first splint strand (320) is hybridized to the at least first left universal adaptor sequence (120) of the library molecule, and a second region of the first splint strand (330) is hybridized to the at least first right universal sequence (130) of the library molecule, thereby circularizing the library molecule to generate a library-splint complex (500). The library-splint complex (500) comprises a first nick between the 5' end of the library molecule and the 3' end of the second splint strand (e.g., FIGS. 1-7). The library-splint complex (500) also comprises a second nick between the 5' end of the second splint strand and the 3' end of the library molecule (e.g., FIGS. 1-7). In some embodiments, the first and second nicks are enzymatically ligatable.

**[0290]** In some embodiment, in the methods for forming a plurality of library-splint complexes (500), the first region of the first splint strand (320) can hybridize to a sense or anti-sense strand of a double-stranded nucleic acid library molecule. In the library-splint complex (500), the second region of the first splint strand (330) can hybridize to a sense or anti-sense strand of a double-stranded nucleic acid library molecule. The double-stranded nucleic acid library molecule can be denatured to generate the single-stranded sense and anti-sense library strands.

**[0291]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the second splint strand (400) does not hybridize to the sequence of interest (110), and the internal region of the first splint strand (310) does not hybridize to the sequence of interest (110).

**[0292]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the first region of the first splint strand (320) does not hybridize to the sequence of interest (110), and the second region of the first splint strand (330) does not hybridize to the sequence of interest (110).

**[0293]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the 5' end of the single-stranded library molecule (100) is phosphorylated or lacks a phosphate group. In some embodiments, the 3' end of the single-stranded library molecule includes a terminal 3' OH group or a terminal 3' blocking group.

**[0294]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), 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) can further comprise additional left and/or right universal adaptor sequences.

**[0295]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the nucleic acid library molecule (100) further comprises a first left index sequence (160). In some embodiments, the nucleic acid library molecule (100) further comprises a first right index sequence (170). In some embodiments, the first left index sequence (160) comprises a sample index sequence. In some embodiments, the first right index sequence (170) comprises another sample index sequence. The sample index sequences can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay. A list of exemplary first left index sequences (160) and first right index sequences (170) is provided in Table 1 at FIGS. 23A-23F. The first left index sequence (160) can include a random sequence (e.g., NNN) or lack a random sequence. The first right index sequence (170) can include a random sequence (e.g., NNN) or lack a random sequence.

**[0296]** Multiplex workflows are enabled by preparing sample-indexed libraries using one or both index sequences (e.g., left and/or right index sequences). The first left index sequences (160) and/or first right index sequences (170) can be employed to prepare separate sample-indexed libraries using input nucleic acids isolated from different sources. The sample-indexed libraries can be pooled together to generate a multiplex library mixture, and the pooled libraries can be amplified and/or sequenced. The sequences of the insert region along with the first left index sequence (160) and/or first right index sequence (170) can be used to identify the source of the input nucleic acids. In some embodiments, any number of sample-indexed libraries can be pooled together, for example 2-10, or 10-50, or 50-100, or 100-200, or more than 200 sample-indexed libraries can be pooled. Exemplary nucleic acid sources include naturally-occurring, recombinant, or chemically-synthesized sources. Exemplary nucleic acid sources include single cells, a plurality of cells, tissue, biological fluid, environmental sample or whole organism. Exemplary nucleic acid sources include fresh, frozen, fresh-frozen or archived sources (e.g., formalin-fixed paraffin-embedded; FFPE). The skilled artisan will recognize that the nucleic acids can be isolated from many other sources. The nucleic acid library molecules can be prepared in single-stranded or double-stranded form.

**[0297]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), 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). In some embodiments, the first left unique identification sequence (180) and the first right unique identification sequence (190) each comprise a sequence that is used to uniquely identify an individual sequence of interest (e.g., insert sequence) to which the unique adaptors are appended in a population of other sequence of interest molecules. In some embodiments, the first left unique identification sequence (180) and/or the first right unique identification sequence (190) can be used for molecular tagging.

**[0298]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the nucleic acid library molecule (100) comprises any one or any combination of two or more: a first left universal adaptor sequence (120); a second left universal adaptor sequence (140); a first left index sequence (160); a first left unique identification sequence (180); a first right universal adaptor sequence (130); a second right universal adaptor sequence (150); a first right index sequence (170); and/or a first right unique identification sequence (190).

**[0299]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the first left universal adaptor sequence (120) and/or the second left universal adaptor sequence (140), comprises: a universal binding sequence for a forward or reverse sequencing primer; a universal binding sequence for a first or second surface primer; a universal binding sequence for a forward or reverse amplification primer; and/or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the nucleic acid library molecule (100) can further comprise additional left universal adaptor sequences.

**[0300]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the first right universal adaptor sequence (130) and/or the second right universal adaptor sequence (150), comprises: a universal binding sequence for a forward or reverse sequencing primer; a universal binding sequence for a first or second surface primer; a universal binding sequence for a forward or reverse amplification primer; and/or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the nucleic acid library molecule (100) can further comprise additional right universal adaptor sequences.

**[0301]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the nucleic acid library molecule (100) further comprises at least one junction adaptor sequence located between any of the universal adaptor sequences described herein (e.g., see FIG. 7). For example, a first left junction adaptor sequence (125) can be

located between the first left universal adaptor sequence (120) and the first left index sequence (160). A second left junction adaptor sequence (165) can be located between the first left index sequence (160) and the second left universal adaptor sequence (140). A third junction adaptor sequence (145) can be located between the second left universal adaptor sequence (140) and the sequence-of-interest (110). A first right junction adaptor sequence (135) can be located between the first right universal sequence (130) and the first right index sequence (170). A second right junction adaptor sequence (175) can be located between the first right index sequence (170) and the second right universal adaptor sequence (150). A third right junction adaptor sequence (155) can be located between the second right universal adaptor sequence (150) and the sequence-of-interest (110). In some embodiments, the nucleic acid library molecule (100) further comprises at least one and up to ten appended universal adaptor sequences located 5' (upstream) to the first left universal adaptor sequence (120) (e.g., see FIG. 7). In some embodiments, the nucleic acid library molecule (100) further comprises at least one and up to ten appended universal adaptor sequences located 3' (downstream) to the first right universal sequence (130) (e.g., see FIG. 7). Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise any sequence and can be 3-60 nucleotides in length. Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a universal sequence or a unique sequence. Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a binding sequence for an amplification primer, a sequencing primer or a compaction oligonucleotide. Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a binding sequence for an immobilized surface primer (e.g., capture primer). Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a sample index sequence. Any of the junction adaptor sequences and/or appended universal adaptor sequences comprise a unique identification sequence. Any of the junction adaptor sequences and/or appended universal adaptor sequences, particularly junction adaptor sequence (145) comprises a Tn5 transposon-end sequence 5'-AGATGTGTATAAGAGACAG-3' (SEQ ID NO:211). Any of the junction adaptor sequences and/or appended universal adaptor sequences, particularly junction adaptor sequence (155) comprises a Tn5 transposon-end sequence 5'-CTGTCTCTTATACACATCT-3' (SEQ ID NO:212). The Tn5 transposon-end sequences can be introduced into the library molecule (100) via a transposase-mediated reaction which includes contacting double-stranded input DNA (e.g., genomic DNA) with a Tn-5 type transposase enzyme, and a double-stranded oligonucleotide comprising the Tn transposon-end sequence (SEQ ID

NO:211) linked to a universal adaptor sequence or a sample index sequence under a condition that is suitable to form a transposon synaptic complex. In the double-stranded oligonucleotide, the Tn transposon-end sequence (SEQ ID NO:211) can be located 5' or 3' relative to the universal adaptor sequence or a sample index sequence.

**[0302]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the second splint strand (400) comprises at least two sub-regions, including a first and second sub-region (e.g., FIGS. 2 and 3). The first sub-region comprises a universal binding sequence for a third surface primer, and the second sub-region comprises a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize to (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the second splint strand (400) further comprises an optional third sub-region which includes a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN) (e.g., FIG. 3). In some embodiments, the second splint strand (400) comprises only one sub-region and lacks a second and third sub-region, where the first sub-region comprises a sample index sequence having 5-20 bases. In some embodiments, the sample index sequence can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay. In some embodiments, the unique identification sequence comprises a random sequence. The unique identification sequence can be designed to exhibit reduced or no hybridization to the first, second, third and fourth surface primers. An exemplary arrangement of the sub-regions in the second splint strand (400), in a 5' to 3' orientation comprises: 5' - [second sub-region] - [first sub-region] - 3'. Another exemplary arrangement of the sub-regions in the second splint strand (400), in a 5' to 3' orientation comprises: 5' - [third sub-region] - [second sub-region] - [first sub-region] - 3'. In some embodiments, the second splint strand (400) can be 20-100 nucleotides in length, or 30-80 nucleotides in length, or 40-60 nucleotides in length. In some embodiments, the second splint strands (400) comprise one or more phosphorothioate linkage at the 5' and/or 3' ends to confer exonuclease resistance. In some embodiments, the second splint strands (400) comprise one or more phosphorothioate linkage at an internal position to confer endonuclease resistance. In some embodiments, the second splint strands (400) comprise one or more 2'-O-methylcytosine bases at the 5' and/or 3' end, or at an internal position. In some embodiments, the 5' end of the second splint strand (400) is phosphorylated or non-phosphorylated. In some embodiments, the 3' end of the second splint strand (400) comprises a terminal 3' OH group or a terminal 3' blocking group.

**[0303]** In some embodiments, in the methods for forming a plurality of library-splint complexes (500), the first splint strand (300) includes an internal region (310) which comprises at least two sub-regions, including a fourth and fifth sub-region (e.g., FIGS. 2 and 3). The fourth sub-region hybridizes to the first sub-region of the second splint strand (400). The fifth sub-region hybridizes to the second sub-region of the second splint strand (400). The fourth and fifth sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers. In some embodiments, the internal region (310) of the first splint strand further comprises an optional sixth sub-region which hybridizes to the third sub-region of the second splint strand (400) (e.g., FIG. 3). An exemplary arrangement of the sub-regions of the first splint strand (300), in a 5' to 3' orientation comprises: 5' – [fourth sub-region] – [fifth sub-region] – 3'. Another exemplary arrangement of the sub-regions of the first splint strand (300), in a 5' to 3' orientation comprises: 5' – [fourth sub-region] – [fifth sub-region] – [sixth sub-region] – 3'. In some embodiments, the first splint strand (300) can be 50-150 nucleotides in length, or 60-100 nucleotides in length, or 70-90 nucleotides in length. In some embodiments, the first splint strands (300) comprise one or more phosphorothioate linkage at the 5' and/or 3' ends to confer exonuclease resistance. In some embodiments, the first splint strands (300) comprise one or more phosphorothioate linkage at an internal position to confer endonuclease resistance. In some embodiments, the first splint strands (300) comprise one or more 2'-O-methylcytosine bases at the 5' and/or 3' end, or at an internal position.

**[0304]** The present disclosure provides methods for forming a plurality of library-splint complexes (500) comprising: **(a)** providing a plurality of double-stranded splint adaptors (200) wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the first splint strand (300) comprises regions arranged in a 5' to 3' order a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the second splint strand comprises regions arranged in a 5' to 3' order **(i)** a second sub-region having a universal binding sequence for a fourth surface primer, and **(ii)** a first sub-region having a universal binding sequence for a third surface primer. The methods for forming a plurality of library-splint complexes (500) further comprises **step (b):** hybridizing the plurality of double-stranded splint adaptors with a plurality of single-stranded nucleic acid library molecules (100) wherein individual library molecules 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; **(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 (130), wherein the hybridizing is conducted under a condition suitable to hybridize the first splint strand (300) to the library molecule (100) thereby circularizing the library molecule to generate a library-splint complex (500), such that the first region (320) of the first splint strand is hybridized to the binding sequence for the first surface primer (120), and the third region (330) of the first splint strand is hybridized to the binding sequence for the second surface primer (130), wherein the library-splint complex (500) comprises a first nick between the 5' end of the library molecule and the 3' end of the second splint strand (300), wherein the library-splint complex (500) comprises a second nick between the 5' end of the second splint strand (300) and the 3' end of the library molecule (100), and wherein the first and second nicks are enzymatically ligatable. In some embodiments, the plurality of single-stranded nucleic acid library molecules (100) further comprise a first left index sequence (160) and/or a first right index sequence (170) (e.g., see FIG. 5). A list of exemplary first left index sequences (160) and first right index sequences (170) is provided in Table 1 at FIGS. 23A-23F. In some embodiments, the first left index sequences (160) include or lack a short random sequence (e.g., NNN). In some embodiments, the first right index sequences (170) include or lack a short random sequence (e.g., NNN). In some embodiments, 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) each comprising a sequence that is used to uniquely identify an individual sequence of interest (e.g., insert sequence) to which the unique adaptors are appended in a population of other sequence of interest molecules. In some embodiments, the first left unique identification sequence (180) and/or the first right unique identification sequence (190) can be used for molecular tagging. (e.g., see FIG. 6).

**[0305]** Multiplex workflows are enabled by preparing sample-indexed libraries using one or both index sequences (e.g., left and/or right index sequences). The first left index sequences (160) and/or first right index sequences (170) can be employed to prepare separate sample-indexed libraries using input nucleic acids isolated from different sources. The sample-indexed libraries can be pooled together to generate a multiplex library mixture, and the pooled libraries can be amplified and/or sequenced. The sequences of the insert region along with the first left index sequence (160) and/or first right index sequence (170) can be used to

identify the source of the input nucleic acids. In some embodiments, any number of sample-indexed libraries can be pooled together, for example 2-10, or 10-50, or 50-100, or 100-200, or more than 200 sample-indexed libraries can be pooled. Exemplary nucleic acid sources include naturally-occurring, recombinant, or chemically-synthesized sources. Exemplary nucleic acid sources include single cells, a plurality of cells, tissue, biological fluid, environmental sample or whole organism. Exemplary nucleic acid sources include fresh, frozen, fresh-frozen or archived sources (e.g., formalin-fixed paraffin-embedded; FFPE). The skilled artisan will recognize that the nucleic acids can be isolated from many other sources. The nucleic acid library molecules can be prepared in single-stranded or double-stranded form.

**[0306]** In some embodiments, 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) (e.g., see FIGS. 5 and 6). In some embodiments, the first left unique identification sequence (180) and the first right unique identification sequence (190) each comprise a sequence that is used to uniquely identify an individual sequence of interest (e.g., insert sequence) to which the unique adaptors are appended in a population of other sequence of interest molecules. In some embodiments, the first left unique identification sequence (180) and/or the first right unique identification sequence (190) can be used for molecular tagging.

**[0307]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the first sub-region of the second splint strand (400) comprises the sequence 5'-CATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:200). In some embodiments, the second sub-region of the second splint strand (400) comprises the sequence 5'-AGTCGTCGCAGCCTCACCTGATC-3' (SEQ ID NO:201). In some embodiments, the second splint strand (400) comprises a first and second sub-region comprising the sequence 5'-

AGTCGTCGCAGCCTCACCTGATCCATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:202). See FIG. 10. In some embodiments, the 5' end of the second splint strand (400) can be phosphorylated or non-phosphorylated.

**[0308]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the first region of the first splint strand (320) includes a first universal adaptor sequence which comprises a universal binding sequence (or a complementary sequence thereof) for a first surface primer, where the first region (320) comprises the sequence 5'-TCGGTGGTCGCCGTATCATT-3' (SEQ ID NO:193). For

example, the first region of the first splint strand (320) can hybridize to a P5 surface primer or a complementary sequence of the P5 surface primer. For example, the P5 surface primer comprises the sequence

5'-AATGATACGGCGACCACCGA-3' (SEQ ID NO:203; short P5), or the P5 surface primer comprises the sequence 5'-AATGATACGGCGACCACCGAGATC-3' (SEQ ID NO:194; long P5). In some embodiments, the second region of the first splint strand (330) includes a second universal adaptor sequence which comprises a universal binding sequence (or a complementary sequence thereof) for a second surface primer, where the second region (330) comprises the sequence 5'-CAAGCAGAAGACGGCATACTGA-3' (SEQ ID NO:195). For example, the second region of the first splint strand (330) can hybridize to a P7 surface primer or a complementary sequence of the P7 surface primer. For example, the P7 surface primer comprises the sequence 5'-CAAGCAGAAGACGGCATACTGA-3' (SEQ ID NO:195; short P7), or the P7 surface primer comprises the sequence 5'-CAAGCAGAAGACGGCATACTGAGAT-3' (SEQ ID NO:196; long P7). In some embodiments, the first splint strand (300) includes an internal region (310) which comprises a fourth sub-region having the sequence

5'-ACCCTGAAAGTACGTGCATTACATG-3' (SEQ ID NO:197). In some embodiments, the first splint strand (300) includes an internal region (310) which comprises a fifth sub-region having the sequence 5'-GATCAGGTGAGGCTGCGACGACT-3' (SEQ ID NO:198). In some embodiments, the first splint strand (300) comprises a first region (320), an internal region (310) having a fourth and fifth sub-region, and a second region (330), having the sequence 5'-

TCGGTGGTCGCCGTATCATTACCCTGAAAGTACGTGCATTACATGGATCAGGTGAGGCTGCGACGACTCAAGCAGAAGACGGCATACTGA-3' (SEQ ID NO:199). See FIG.

10. In some embodiments, the 5' end of the first splint strand (300) can be phosphorylated or non-phosphorylated. In some embodiments, the first sub-region of the second splint strand (400) can hybridize to the fourth sub-region of the first splint strand (300). In some embodiments, the second sub-region of the second splint strand (400) can hybridize to the fifth sub-region of the first splint strand (300).

**[0309]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the first region of the first splint strand (320) comprises a sequence that can bind a first left universal adaptor sequence (120) of a library molecules, where the first region of the first splint strand (320) comprises the sequence 5'-

ACCCTGAAAGTACGTGCATTACATG -3' (SEQ ID NO:197) or a complementary sequence thereof.

**[0310]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the second region of the first splint strand (330) comprises a sequence that can bind a first right universal adaptor sequence (130) of a library molecules, where the second region of the first splint strand (330) comprises the sequence 5'- GATCAGGTGAGGCTGCGACGACT -3' (SEQ ID NO:198) or a complementary sequence thereof.

**[0311]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the library molecule includes a left universal binding sequence (120) which binds the first region of the first splint strand (320), where the left universal binding sequence (120) comprises the sequence 5'- AATGATACGGCGACCACCGA-3' (SEQ ID NO:203).

**[0312]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the library molecule includes a left universal binding sequence (120) which binds the first region of the first splint strand (320), where the left universal binding sequence (120) comprises the sequence 5'- CATGTAATGCACGTACTTTCAGGGT -3' (SEQ ID NO:200) or a complementary sequence thereof.

**[0313]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the library molecule includes a left universal binding sequence for a sequencing primer (140) where the left universal binding sequence comprises the sequence 5'- ACACTCTTTCCTACACGACGCTCTTCCGATCT -3' (SEQ ID NO:204).

**[0314]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the library molecule includes a left universal binding sequence for a sequencing primer (140) where the left universal binding sequence comprises the sequence 5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG -3' (SEQ ID NO:207).

**[0315]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the library molecule includes a left universal binding sequence for a sequencing primer (140) where the left universal binding sequence comprises the sequence 5'- CGTGCTGGATTGGCTCACCAGACACCTTCCGACAT -3' (SEQ ID NO:208).

**[0316]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the library molecule includes a left universal binding sequence for a sequencing primer (150) where the left universal binding sequence comprises the sequence 5'-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3' (SEQ ID NO:205).

**[0317]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the library molecule includes a left universal binding sequence for a sequencing primer (150) where the left universal binding sequence comprises the sequence 5'-CTGTCTCTTATACACATCTCCGAGCCCACGAGAC-3' (SEQ ID NO:209).

**[0318]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the library molecule includes a left universal binding sequence for a sequencing primer (150) where the left universal binding sequence comprises the sequence 5'-ATGTCGGAAGGTGTGCAGGCTACCGCTTGTCAACT-3' (SEQ ID NO:210).

**[0319]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the library molecule includes a right universal binding sequence (130) which binds the first region of the first splint strand (330), where the right universal binding sequence (130) comprises the sequence 5'-TCGTATGCCGTCTTCTGCTTG-3' (SEQ ID NO:206).

**[0320]** In some embodiments, in any of the methods for forming a plurality of library-splint complexes (500) described herein, the library molecule includes a right universal binding sequence (130) which binds the first region of the first splint strand (330), where the right universal binding sequence (130) comprises the sequence 5'-AGTCGTCGCAGCCTCACCTGATC-3' (SEQ ID NO:201) or a complementary sequence thereof.

**[0321]** In some embodiments, any of the methods for forming a plurality of library-splint complexes (500) described herein can further comprises at least one enzymatic reaction, including a phosphorylation reaction, ligation reaction and/or exonuclease reaction. The enzymatic reactions can be conducted sequentially or essentially simultaneously. The enzymatic reactions can be conducted in a single reaction vessel. Alternatively, a first enzymatic reaction can be conducted in a first reaction vessel, then transferred to a second reaction vessel where the second enzymatic reaction is conducted, then transferred to a third reaction vessel where the third enzymatic reaction is conducted.

**[0322]** In some embodiments, any of the methods for forming a plurality of library-splint complexes (500) described herein further comprise conducting separate and sequential phosphorylation and ligation reactions which are conducted in separate reaction vessels. In some embodiments, the methods for forming a plurality of library-splint complexes (500) further comprise **step (c1)**: contacting in a first reaction vessel the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a T4 polynucleotide kinase enzyme under a condition suitable to phosphorylate the 5' ends of the plurality of double-stranded splint adaptors (200) and/or the plurality of single-stranded nucleic acid library molecules (100); and transferring the phosphorylation reaction to a second reaction vessel. In some embodiments, the methods for forming a plurality of library-splint complexes (500) further comprise **step (d1)**: contacting in the second reaction vessel the plurality of phosphorylated double-stranded splint adaptors (200) and the plurality of phosphorylated single-stranded nucleic acid library molecules (100) with a ligase, under a condition suitable to enzymatically ligate the first and second nicks, thereby generating a plurality of covalently closed circular library molecules (600) each hybridized to the first splint strand (300). In some embodiments, the ligase enzyme comprises T7 DNA ligase, T3 ligase, T4 ligase, or Taq ligase.

**[0323]** In some embodiments, any of the methods for forming a plurality of library-splint complexes (500) described herein further comprise conducting sequential phosphorylation and ligation reactions which are conducted sequentially in the same reaction vessel. In some embodiments, the methods for forming a plurality of library-splint complexes (500) further comprise **step (c2)**: contacting in a first reaction vessel the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a T4 polynucleotide kinase enzyme under a condition suitable to phosphorylate the 5' ends of the plurality of double-stranded splint adaptors (200) and the plurality of single-stranded nucleic acid library molecules (100). In some embodiments, the methods for forming a plurality of library-splint complexes (500) further comprise **step (d2)**: contacting in the same first reaction vessel the phosphorylated double-stranded splint adaptors (200) and the phosphorylated single-stranded nucleic acid library molecules (100) with a ligase under a condition suitable to enzymatically ligate the first and second nicks, thereby generating a plurality of covalently closed circular library molecules (600) each hybridized to the first splint strand (300). In some embodiments, the ligase enzyme comprises T7 DNA ligase, T3 ligase, T4 ligase, or Taq ligase.

**[0324]** In some embodiments, any of the methods for forming a plurality of library-splint complexes (500) described herein further comprise conducting essentially simultaneous phosphorylation and ligation reactions which are conducted together in the same reaction vessel. In some embodiments, the methods for forming a plurality of library-splint complexes (500) further comprise **step (c3)**: contacting in a first reaction vessel the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a **(i)** T4 polynucleotide kinase enzyme and **(ii)** a ligase enzyme, under a condition suitable to phosphorylate the 5' ends of the plurality of double-stranded splint adaptors (200) and the plurality of single-stranded nucleic acid library molecules (100), and the conditions are suitable to enzymatically ligate the first and second nicks, thereby generating a plurality of covalently closed circular library molecules (600) each hybridized to the first splint strand (300). In some embodiments, the ligase enzyme comprises T7 DNA ligase, T3 ligase, T4 ligase, or Taq ligase.

**[0325]** In some embodiments, any of the methods for forming a plurality of library-splint complexes (500) described herein further comprise the optional step of enzymatically removing the plurality of first splint strands (300) from the plurality of covalently closed circular library molecules (600), which comprises: contacting the plurality of covalently closed circular library molecules (600) with at least one exonuclease enzyme to remove the plurality of first splint strands (300) and retaining the plurality of covalently closed circular library molecules (600). In some embodiments, the exonuclease reaction can be conducted in the same reaction buffer used to conduct the phosphorylation and/or ligation reactions, or in a different reaction buffer. In some embodiments, the exonuclease reaction can be conducted in a third reaction vessel after conducting the phosphorylation reaction in the first reaction vessel (c1), and conducting the ligation reaction in the second reaction vessel (d1). In some embodiments, the exonuclease reaction can be conducted in the first reaction vessel after conducting the phosphorylation reaction in the first reaction vessel (c2), and conducting the sequential ligation reaction in the first reaction vessel (d2). In some embodiments, the exonuclease reaction can be conducted in the first reaction vessel after conducting the essentially simultaneous phosphorylation and ligation reactions in the first reaction vessel (c3). In some embodiments, the at least one exonuclease enzyme comprises any combination of two or more of exonuclease I, thermolabile exonuclease I and/or T7 exonuclease.

## **Methods for Rolling Circle Amplification**

**[0326]** The present disclosure provides methods for conducting rolling circle amplification reaction on the covalently closed circular library molecules (600). The rolling circle amplification reaction can be conducted after the phosphorylation and ligation reactions, or after the ligation reaction. In some embodiments, the rolling circle amplification reaction can be conducted on covalently closed circular library molecules (600) that are no longer hybridized to the first splint strands (300) following the exonuclease reaction. In some embodiments, the rolling circle amplification reaction can be conducted on covalently closed circular library molecules (600) that are hybridized to the first splint strands (300). In some embodiments, the covalently closed circular library molecules (600) can be distributed onto a support and then be subjected to rolling circle amplification reaction. In some embodiments, the covalently closed circular library molecules (600) can be subjected to rolling circle amplification reaction in-solution and then distributed onto a support. In some embodiments, the rolling circle amplification reactions can employ the retained first splint strand (300) as an amplification primer, or the first splint strand (300) can be removed (e.g., via exonuclease digestion) and replaced with a soluble amplification primer.

#### **On-Support Rolling Circle Amplification**

**[0327]** In some embodiments, the methods for conducting rolling circle amplification reaction on a plurality of covalently closed circular library molecules which lack hybridized first splint strands (300), wherein individual covalently closed circular library molecules (600) in the plurality comprise a second splint strand region (400) which includes a universal binding sequence for a fourth surface primer, the method comprises **(a)**: distributing the plurality of covalently closed circular library molecules (600) onto a support having a plurality of the fourth surface primers immobilized on the support, under a condition suitable for hybridizing individual covalently closed circular library molecules (600) to individual immobilized fourth surface primers thereby immobilizing the plurality of covalently closed circular library molecules (600).

**[0328]** In some embodiments, the plurality of the fourth surface primers immobilized on the support comprise the sequence 5'-GATCAGGTGAGGCTGCGACGACT-3' (SEQ ID NO:198). Individual fourth surface primers can hybridize to a covalently closed circular library molecule (600) having a second splint strand region (400) which includes a universal binding sequence for a fourth surface primer, where the universal binding sequence for a

fourth surface primer comprises a second sub-region which comprises the sequence 5'-AGTCGTCGCAGCCTCACCTGATC-3' (SEQ ID NO:201).

**[0329]** In some embodiments, the plurality of covalently closed circular library molecules (600) can be distributed onto a support that is coated with one or more compounds to produce a passivated layer on the support (e.g., FIG. 11). In some embodiments, the passivated layer forms a porous or semi-porous layer. In some embodiments, the surface primer, concatemer template molecule and/or polymerase, can be attached to the passivated layer for immobilization to the support. In some embodiments, the support comprises a low non-specific binding surface that enable improved nucleic acid hybridization and amplification performance on the support. In general, the support may comprise one or more layers of a covalently or non-covalently attached low-binding, chemical modification layers, e.g., silane layers, polymer films, and one or more covalently or non-covalently attached oligonucleotides that can be used for immobilizing a plurality of nucleic acid concatemer molecules to the support. In some embodiments, the support can comprise a functionalized polymer coating layer covalently bound at least to a portion of the support via a chemical group on the support, a primer grafted to the functionalized polymer coating, and a water-soluble protective coating on the primer and the functionalized polymer coating. In some embodiments, the functionalized polymer coating comprises a poly(N-(5-azidoacetamidylpentyl)acrylamide-co-acrylamide (PAZAM). In some embodiments, the support comprises a surface coating having at least one hydrophilic polymer coating layer and at least one layer of a plurality of oligonucleotides. The hydrophilic polymer coating layer can comprise polyethylene glycol (PEG). The hydrophilic polymer coating layer can comprise branched PEG having at least 4 branches. In some embodiments, the low non-specific binding coating has a degree of hydrophilicity which can be measured as a water contact angle, where the water contact angle is no more than 45 degrees. In some embodiments, the density of the covalently closed circular library molecules (600) immobilized to the support or immobilized to the coating on the support is about  $10^2$ - $10^6$  per  $\text{mm}^2$ , or about  $10^6$ - $10^9$  per  $\text{mm}^2$ , or about  $10^9$ - $10^{12}$  per  $\text{mm}^2$ . In some embodiments, the plurality of covalently closed circular library molecules (600) are immobilized to the support or immobilized to the coating on the support at pre-determined sites on the support (or the coating on the support), or immobilized to the coating on the support at random sites on the support (or the coating on the support).

**[0330]** In some embodiments, the distributing of **step (a)** can be conducted in the presence of a high-efficiency hybridization buffer which comprises: (i) a first polar aprotic solvent

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

**[0331]** In some embodiments, the methods for conducting rolling circle amplification reaction further comprises **step (b)**: contacting the plurality of immobilized covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of fourth surface primers as immobilized amplification primers and the plurality of covalently closed circular library molecules (600) as template molecules, thereby generating a plurality of nucleic acid concatemer molecules immobilized to the fourth surface primers. In some embodiments, the plurality of nucleotides comprises any combination of two or more of dATP, dGTP, dCTP, dTTP and/or dUTP. In some embodiments, individual immobilized concatemers are covalently joined to individual fourth surface primers. In some embodiments, individual covalently closed circular library molecules (600) in the plurality comprise a second splint strand region (400) which also include a universal binding sequence for a fourth surface primer so that the rolling circle amplification reaction generates concatemer molecules having multiple copies of universal binding sequences for third and fourth surface primers. In some embodiments, the method comprises distributing the covalently closed circular library molecules (600) onto a support comprising a plurality of immobilized third and fourth surface primers, conducting a rolling circle amplification reaction to generate concatemer molecules under a condition suitable for hybridizing at least one second splint strand region (400) of the concatemer molecules to immobilized third and fourth surface primers thereby pinning down at least one portion of the

concatemer molecules to the support. In some embodiments, the immobilized concatemers can be subjected to sequencing reactions.

**[0332]** In some embodiments, the plurality of the fourth surface primers immobilized on the support comprise the sequence 5'-CATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:200) or a complementary sequence thereof). Individual fourth surface primers can hybridize to a portion of the concatemer molecules having a second splint strand region (400) which includes a universal binding sequence for a fourth surface primer (or a complementary sequence thereof), where the universal binding sequence for the fourth surface primer comprises a first sub-region which comprises the sequence 5'-CATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:200) or a complementary sequence thereof.

### **In-Solution Rolling Circle Amplification Using Soluble Amplification Primers**

**[0333]** In some embodiments, the methods for conducting rolling circle amplification reaction on a plurality of covalently closed circular library molecules which lack hybridized first splint strands (300), wherein individual covalently closed circular library molecules (600) in the plurality comprise a first (120) or second (140) left universal adaptor sequence of a universal binding sequence for a forward amplification primer, and wherein individual covalently closed circular library molecules (600) in the plurality comprise a second splint strand region (400) which includes a universal binding sequence for a fourth surface primer, the method comprises **(a)** hybridizing in solution a plurality of soluble forward amplification primers to the first or second left universal adaptor sequence which comprise the universal binding sequence for a forward amplification primer; and **(b)** conducting a first rolling circle amplification reaction by contacting the plurality of covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction in solution using the plurality of forward amplification primers and the plurality of covalently closed circular library molecules (600) as template molecules, thereby generating a plurality of nucleic acid concatemer molecules which are still hybridized to covalently closed circular library molecules (600). In some embodiments, the methods for conducting rolling circle amplification reaction further comprises step **(c)**: distributing the plurality of concatemer molecules onto a support having a plurality of the fourth surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to the plurality of the immobilized fourth surface primers thereby immobilizing the plurality of

concatemer molecules. The plurality of immobilized concatemer molecules are still hybridized to covalently closed circular library molecules (600). In some embodiments, the methods for conducting rolling circle amplification reaction further comprises step **(d)**: contacting the immobilized plurality of concatemer molecules with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a second rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules. In some embodiments, the first and/or the second rolling circle amplification reactions can be conducted with a plurality of nucleotides which comprise any combination of two or more of dATP, dGTP, dCTP, dTTP and/or dUTP. In some embodiments, individual immobilized concatemers are hybridized to individual fourth surface primers. In some embodiments, individual covalently closed circular library molecules (600) in the plurality comprise a second splint strand region (400) which also include a universal binding sequence for a fourth surface primer so that the first rolling circle amplification reaction in solution generates concatemer molecules having multiple copies of universal binding sequences for third and fourth surface primers. In some embodiments, the method comprises distributing the concatemer molecules onto a support comprising a plurality of immobilized third and fourth surface primers, and incubating the concatemer molecules under a condition suitable for hybridizing at least one second splint strand region (400) of the concatemer molecules to immobilized third and fourth surface primers thereby pinning down at least one portion of the concatemer molecules to the support. In some embodiments, the immobilized concatemers can be subjected to sequencing reactions.

**[0334]** In some embodiments, the plurality of concatemer molecules of step (c) can be distributed onto a support comprising a plurality of the fourth surface primers immobilized on the support comprise the sequence 5'-GATCAGGTGAGGCTGCGACGACT-3' (SEQ ID NO:198). Individual fourth surface primers can hybridize to a portion of a concatemer molecule having a universal binding sequence for a fourth surface primer, where the universal binding sequence for a fourth surface primer comprises the sequence 5'-AGTCGTCGCAGCCTCACCTGATC-3' (SEQ ID NO:201 or a complementary sequence thereof).

**[0335]** In some embodiments, the plurality of concatemer molecules of step (c) can be distributed onto a support comprising a plurality of the fourth surface primers immobilized on the support comprise the sequence 5'-CATGTAATGCACGTACTTTCAGGGT-3' (SEQ ID NO:200 or a complementary sequence thereof). Individual fourth surface primers can

hybridize to a portion of the concatemer molecules having a universal binding sequence for a fourth surface primer (or a complementary sequence thereof), where the universal binding sequence for the fourth surface primer comprises the sequence 5'-

CATGTAATGCACGTACTTTCAGGGT -3' (SEQ ID NO:200) or a complementary sequence thereof.

**[0336]** In some embodiments, the plurality of concatemer molecules of step (c) can be distributed onto a support that is coated with one or more compounds to produce a passivated layer on the support (e.g., FIG. 11). In some embodiments, the passivated layer forms a porous or semi-porous layer. In some embodiments, the surface primer, concatemer template molecule and/or polymerase, can be attached to the passivated layer for immobilization to the support. In some embodiments, the support comprises a low non-specific binding surface that enable improved nucleic acid hybridization and amplification performance on the support. In general, the support may comprise one or more layers of a covalently or non-covalently attached low-binding, chemical modification layers, e.g., silane layers, polymer films, and one or more covalently or non-covalently attached oligonucleotides that can be used for immobilizing a plurality of nucleic acid concatemer molecules to the support. In some embodiments, the support can comprise a functionalized polymer coating layer covalently bound at least to a portion of the support via a chemical group on the support, a primer grafted to the functionalized polymer coating, and a water-soluble protective coating on the primer and the functionalized polymer coating. In some embodiments, the functionalized polymer coating comprises a poly(N-(5-azidoacetamidylpentyl)acrylamide-co-acrylamide (PAZAM). In some embodiments, the support comprises a surface coating having at least one hydrophilic polymer coating layer and at least one layer of a plurality of oligonucleotides. The hydrophilic polymer coating layer can comprise polyethylene glycol (PEG). The hydrophilic polymer coating layer can comprise branched PEG having at least 4 branches. In some embodiments, the low non-specific binding coating has a degree of hydrophilicity which can be measured as a water contact angle, where the water contact angle is no more than 45 degrees. In some embodiments, the density of the concatemer molecules immobilized to the support or immobilized to the coating on the support is about  $10^2$ - $10^6$  per  $\text{mm}^2$ , or about  $10^6$ - $10^9$  per  $\text{mm}^2$ , or about  $10^9$ - $10^{12}$  per  $\text{mm}^2$ . In some embodiments, the plurality of the concatemer molecules are immobilized to the support or immobilized to the coating on the support at pre-determined sites on the support (or the coating on the support), or immobilized to the coating on the support at random sites on the support (or the coating on the support).

**[0337]** In some embodiments, the distributing of step (c) can be conducted in the presence of a high-efficiency hybridization buffer which comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

**[0338]** In some embodiments, the methods for conducting rolling circle amplification reaction on a plurality of covalently closed circular library molecules which lack hybridized first splint strands (300), wherein individual covalently closed circular library molecules (600) in the plurality comprise a first (130) or second (150) right universal adaptor sequence of a universal binding sequence for a forward amplification primer, and wherein individual covalently closed circular library molecules (600) in the plurality comprise a second splint strand region (400) which includes a universal binding sequence for a fourth surface primer, the method comprises **(a)** hybridizing in solution a plurality of soluble forward amplification primers to the first or second right universal adaptor sequence which comprise the universal binding sequence for a forward amplification primer; and **(b)** conducting a first rolling circle amplification reaction by contacting the plurality of covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction in solution using the plurality of forward amplification primers and the plurality of covalently closed circular library molecules (600) as template molecules, thereby generating a plurality of nucleic acid concatemer molecules which are still hybridized to covalently closed circular library molecules (600). In some embodiments, the methods for conducting rolling circle amplification reaction further comprises step **(c)**: distributing the plurality of concatemer molecules onto a support having a plurality of the fourth surface primers immobilized

thereon, under a condition suitable for hybridizing at least a portion of the concatemers to the plurality of the immobilized fourth surface primers thereby immobilizing the plurality of concatemer molecules. The plurality of immobilized concatemer molecules are still hybridized to covalently closed circular library molecules (600). In some embodiments, the methods for conducting rolling circle amplification reaction further comprises step **(d)**: contacting the immobilized plurality of concatemer molecules with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a second rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules. In some embodiments, the first and/or the second rolling circle amplification reactions can be conducted with a plurality of nucleotides which comprise any combination of two or more of dATP, dGTP, dCTP, dTTP and/or dUTP. In some embodiments, individual immobilized concatemers are hybridized to individual fourth surface primers. In some embodiments, individual covalently closed circular library molecules (600) in the plurality comprise a second splint strand region (400) which also include a universal binding sequence for a third surface primer so that the first rolling circle amplification reaction in solution generates concatemer molecules having multiple copies of universal binding sequences for third and fourth surface primers. In some embodiments, the method comprises distributing the concatemer molecules onto a support comprising a plurality of immobilized third and fourth surface primers, and incubating the concatemer molecules under a condition suitable for hybridizing at least one second splint strand region (400) of the concatemer molecules to immobilized third and fourth surface primers thereby pinning down at least one portion of the concatemer molecules to the support. In some embodiments, the immobilized concatemers can be subjected to sequencing reactions.

**[0339]** In some embodiments, the plurality of concatemer molecules of step (c) can be distributed onto a support comprising a plurality of the fourth surface primers immobilized on the support comprise the sequence 5'-GATCAGGTGAGGCTGCGACGACT-3' (SEQ ID NO:198). Individual fourth surface primers can hybridize to a portion of a concatemer molecule having a universal binding sequence for a fourth surface primer, where the universal binding sequence for a fourth surface primer comprises the sequence 5'-AGTCGTCGCAGCCTCACCTGATC-3' (SEQ ID NO:201 or a complementary sequence thereof).

**[0340]** In some embodiments, the plurality of concatemer molecules of step (c) can be distributed onto a support comprising a plurality of the third surface primers immobilized on

the support comprise the sequence 5'-CATGTAATGCACGTACTTTCAGGGT -3' (SEQ ID NO:200 or a complementary sequence thereof). Individual third surface primers can hybridize to a portion of the concatemer molecules having a universal binding sequence for a third surface primer (or a complementary sequence thereof), where the universal binding sequence for the third surface primer comprises the sequence 5'-CATGTAATGCACGTACTTTCAGGGT -3' (SEQ ID NO:200) or a complementary sequence thereof.

**[0341]** In some embodiments, the plurality of concatemer molecules of step (c) can be distributed onto a support that is coated with one or more compounds to produce a passivated layer on the support (e.g., FIG. 11). In some embodiments, the passivated layer forms a porous or semi-porous layer. In some embodiments, the surface primer, concatemer template molecule and/or polymerase, can be attached to the passivated layer for immobilization to the support. In some embodiments, the support comprises a low non-specific binding surface that enable improved nucleic acid hybridization and amplification performance on the support. In general, the support may comprise one or more layers of a covalently or non-covalently attached low-binding, chemical modification layers, e.g., silane layers, polymer films, and one or more covalently or non-covalently attached oligonucleotides that can be used for immobilizing a plurality of nucleic acid concatemer molecules to the support. In some embodiments, the support can comprise a functionalized polymer coating layer covalently bound at least to a portion of the support via a chemical group on the support, a primer grafted to the functionalized polymer coating, and a water-soluble protective coating on the primer and the functionalized polymer coating. In some embodiments, the functionalized polymer coating comprises a poly(N-(5-azidoacetamidylpentyl)acrylamide-co-acrylamide (PAZAM). In some embodiments, the support comprises a surface coating having at least one hydrophilic polymer coating layer and at least one layer of a plurality of oligonucleotides. The hydrophilic polymer coating layer can comprise polyethylene glycol (PEG). The hydrophilic polymer coating layer can comprise branched PEG having at least 4 branches. In some embodiments, the low non-specific binding coating has a degree of hydrophilicity which can be measured as a water contact angle, where the water contact angle is no more than 45 degrees. In some embodiments, the density of the concatemer molecules immobilized to the support or immobilized to the coating on the support is about  $10^2$ - $10^6$  per  $\text{mm}^2$ , or about  $10^6$ - $10^9$  per  $\text{mm}^2$ , or about  $10^9$ - $10^{12}$  per  $\text{mm}^2$ . In some embodiments, the plurality of the concatemer molecules are immobilized to the support or immobilized to the coating on the support at pre-determined sites on the support (or the coating on the support), or

immobilized to the coating on the support at random sites on the support (or the coating on the support).

**[0342]** In some embodiments, the distributing of step (c) can be conducted in the presence of a high-efficiency hybridization buffer which comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(*N*-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

### **In-Solution Rolling Circle Amplification Using First Splint Strands**

**[0343]** In some embodiments, the methods for conducting rolling circle amplification reaction on a plurality of covalently closed circular library molecules which are hybridized to first splint strands (300), wherein individual covalently closed circular library molecules (600) in the plurality comprise a second splint strand region (400) which includes a universal binding sequence for a fourth surface primer, the method comprises **(a)**: contacting in solution the plurality of covalently closed circular library molecules (600) which are hybridized to first splint strands (300) with a plurality of strand-displacing polymerases and a plurality of nucleotides under a condition suitable for conducting a first rolling circle amplification reaction using the first splint strand (300) as an amplification primer thereby generating a plurality of concatemer molecules which are still hybridized to covalently closed circular library molecules (600). See FIG. 10.

**[0344]** In some embodiments, the methods for conducting rolling circle amplification reaction further comprises step **(b)**: distributing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600) onto a support having a plurality of the fourth surface primers immobilized thereon, under a condition suitable for

hybridizing at least a portion of the concatemers to the plurality of the immobilized fourth surface primers thereby immobilizing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600).

**[0345]** In some embodiments, the plurality of concatemer molecules of step (b) can be distributed onto a support comprising a plurality of the fourth surface primers immobilized on the support comprise the sequence 5'-GATCAGGTGAGGCTGCGACGACT-3' (SEQ ID NO:198). Individual fourth surface primers can hybridize to a portion of a concatemer molecule having a universal binding sequence for a fourth surface primer, where the universal binding sequence for a fourth surface primer comprises the sequence 5'-AGTCGTCGCAGCCTCACCTGATC-3' (SEQ ID NO:201 or a complementary sequence thereof).

**[0346]** In some embodiments, the plurality of concatemer molecules of step (b) can be distributed onto a support comprising a plurality of the fourth surface primers immobilized on the support comprise the sequence 5'-CATGTAATGCACGTACTTTCAGGGT -3' (SEQ ID NO:200 or a complementary sequence thereof). Individual fourth surface primers can hybridize to a portion of the concatemer molecules having a universal binding sequence for a fourth surface primer (or a complementary sequence thereof), where the universal binding sequence for the fourth surface primer comprises the sequence 5'-CATGTAATGCACGTACTTTCAGGGT -3' (SEQ ID NO:200) or a complementary sequence thereof.

**[0347]** In some embodiments, the plurality of concatemer molecules of step (b) can be distributed onto a support that is coated with one or more compounds to produce a passivated layer on the support (e.g., FIG. 11). In some embodiments, the passivated layer forms a porous or semi-porous layer. In some embodiments, the surface primer, concatemer template molecule and/or polymerase, can be attached to the passivated layer for immobilization to the support. In some embodiments, the support comprises a low non-specific binding surface that enable improved nucleic acid hybridization and amplification performance on the support. In general, the support may comprise one or more layers of a covalently or non-covalently attached low-binding, chemical modification layers, e.g., silane layers, polymer films, and one or more covalently or non-covalently attached oligonucleotides that can be used for immobilizing a plurality of nucleic acid concatemer molecules to the support. In some embodiments, the support can comprise a functionalized polymer coating layer covalently bound at least to a portion of the support via a chemical group on the support, a primer grafted to the functionalized polymer coating, and a water-soluble protective coating on the

primer and the functionalized polymer coating. In some embodiments, the functionalized polymer coating comprises a poly(N-(5-azidoacetamidylpentyl)acrylamide-co-acrylamide (PAZAM). In some embodiments, the support comprises a surface coating having at least one hydrophilic polymer coating layer and at least one layer of a plurality of oligonucleotides. The hydrophilic polymer coating layer can comprise polyethylene glycol (PEG). The hydrophilic polymer coating layer can comprise branched PEG having at least 4 branches. In some embodiments, the low non-specific binding coating has a degree of hydrophilicity which can be measured as a water contact angle, where the water contact angle is no more than 45 degrees. In some embodiments, the density of the concatemer molecules immobilized to the support or immobilized to the coating on the support is about  $10^2$ - $10^6$  per  $\text{mm}^2$ , or about  $10^6$ - $10^9$  per  $\text{mm}^2$ , or about  $10^9$ - $10^{12}$  per  $\text{mm}^2$ . In some embodiments, the plurality of the concatemer molecules are immobilized to the support or immobilized to the coating on the support at pre-determined sites on the support (or the coating on the support), or immobilized to the coating on the support at random sites on the support (or the coating on the support).

**[0348]** In some embodiments, the distributing of step (b) can be conducted in the presence of a high-efficiency hybridization buffer which comprises: (i) a first polar aprotic solvent having a dielectric constant that is no greater than 40 and having a polarity index of 4-9; (ii) a second polar aprotic solvent having a dielectric constant that is no greater than 115 and is present in the hybridization buffer formulation in an amount effective to denature double-stranded nucleic acids; (iii) a pH buffer system that maintains the pH of the hybridization buffer formulation in a range of about 4-8; and (iv) a crowding agent in an amount sufficient to enhance or facilitate molecular crowding. In some embodiments, the high efficiency hybridization buffer comprises: (i) the first polar aprotic solvent comprises acetonitrile at 25-50% by volume of the hybridization buffer; (ii) the second polar aprotic solvent comprises formamide at 5-10% by volume of the hybridization buffer; (iii) the pH buffer system comprises 2-(N-morpholino)ethanesulfonic acid (MES) at a pH of 5-6.5; and (iv) the crowding agent comprises polyethylene glycol (PEG) at 5-35% by volume of the hybridization buffer. In some embodiments, the high efficiency hybridization buffer further comprises betaine.

**[0349]** In some embodiments, the methods for conducting rolling circle amplification reaction further comprises step (c): contacting the plurality of immobilized concatemer molecules with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a second rolling circle amplification reaction on the

support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules.

**[0350]** In some embodiments, the first and/or the second rolling circle amplification reactions can be conducted with a plurality of nucleotides which comprise any combination of two or more of dATP, dGTP, dCTP, dTTP and/or dUTP. In some embodiments, individual covalently closed circular library molecules (600) in the plurality comprise a second splint strand region (400) which also include a universal binding sequence for a third surface primer so that the first rolling circle amplification reaction in solution generates concatemer molecules having multiple copies of universal binding sequences for third and fourth surface primers. In some embodiments, the method comprises distributing the concatemer molecules onto a support comprising a plurality of immobilized third and fourth surface primers, and incubating the plurality of immobilized nucleic acid concatemer molecules under a condition suitable for hybridizing at least one second splint strand region (400) of the concatemer molecules to the immobilized third and fourth surface primers thereby pinning down at least one portion of the concatemer molecules to the support. In some embodiments, individual immobilized concatemers are hybridized to individual third and fourth surface primers. In some embodiments, the immobilized concatemers can be subjected to sequencing reactions.

### **Methods for Sequencing**

**[0351]** The present disclosure provides methods for sequencing any of the immobilized concatemer molecules described herein. Any of the methods for conducting rolling circle amplification reaction described herein can be used to generate a plurality of concatemer molecules immobilized to a support, and the immobilized concatemers can be subjected to sequencing reactions. In some embodiments, the sequencing reactions employ detectably labeled nucleotide analogs. In some embodiments, the sequencing reactions employ a two-stage sequencing reaction comprising binding detectably labeled multivalent molecules, and incorporating nucleotide analogs. The terms concatemer molecule and template molecule are used interchangeably.

**[0352]** In some embodiments, any of the rolling circle amplification reaction described herein (e.g., RCA conducted on-support or in-solution) can be used to generate immobilized concatemers each containing tandem repeat units of the sequence-of-interest and any adaptor sequences present in the covalently closed circular library molecules (600). For example, the tandem repeat unit comprises: **(i)** a first left universal adaptor sequence (120) having a

binding sequence for a first surface primer, **(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, **(v)** a first right universal adaptor sequence (130) having a binding sequence for a second surface primer, and **(vii)** a first left index sequence (160) and/or a first right index sequence (170) (e.g., see FIGS. 5-7). In some embodiments, the tandem repeat unit further comprises a first left unique identification sequence (180) and/or a first right unique identification sequence (190).

**[0353]** The immobilized concatemer can self-collapse into a compact nucleic acid nanoball. Inclusion of one or more compaction oligonucleotides during the RCA reaction can further compact the size and/or shape of the nanoball. An increase in the number of tandem repeat units in a given concatemer increases the number of sites along the concatemer for hybridizing to multiple sequencing primers (e.g., sequencing primers having a universal sequence) which serve as multiple initiation sites for polymerase-catalyzed sequencing reactions. When the sequencing reaction employs detectably labeled nucleotides and/or detectably labeled multivalent molecules (e.g., having nucleotide units), the signals emitted by the nucleotides or nucleotide units that participate in the parallel sequencing reactions along the concatemer yields an increased signal intensity for each concatemer. Multiple portions of a given concatemer can be simultaneously sequenced. Furthermore, a plurality of binding complexes can form along a particular concatemer molecule, each binding complex comprising a sequencing polymerase bound to a multivalent molecule wherein the plurality of binding complexes remain stable without dissociation resulting in increased persistence time which increases signal intensity and reduces imaging time.

### **Methods for Sequencing using Nucleotide Analogs**

**[0354]** The present disclosure provides methods for sequencing, comprising **step (a)**: contacting a sequencing polymerase to (i) a nucleic acid concatemer molecule and (ii) a nucleic acid primer, wherein the contacting is conducted under a condition suitable to bind the sequencing polymerase to the nucleic acid concatemer molecule which is hybridized to the nucleic acid primer, wherein the nucleic acid concatemer molecule hybridized to the nucleic acid primer forms the nucleic acid duplex. In some embodiments, the sequencing polymerase comprises a recombinant mutant sequencing polymerase. In some embodiments, the primer comprises a 3' extendible end.

**[0355]** In some embodiments, the methods for sequencing further comprise **step (b)**: contacting the sequencing polymerase with a plurality of nucleotides under a condition suitable for binding at least one nucleotide to the sequencing polymerase which is bound to the nucleic acid duplex and suitable for polymerase-catalyzed nucleotide incorporation. In some embodiments, the sequencing polymerase is contacted with the plurality of nucleotides in the presence of at least one catalytic cation comprising magnesium and/or manganese. In some embodiments, the plurality of nucleotides comprises at least one nucleotide analog having a chain terminating moiety at the sugar 2' or 3' position. In some embodiments, the plurality of nucleotides comprises at least one nucleotide that lacks a chain terminating moiety.

**[0356]** In some embodiments, the methods for sequencing further comprise **step (c)**: incorporating at least one nucleotide into the 3' end of the extendible primer under a condition suitable for incorporating the at least one nucleotide. In some embodiments, the suitable conditions for nucleotide binding the polymerase and for incorporation the nucleotide can be the same or different. In some embodiments, conditions suitable for incorporating the nucleotide comprise inclusion of at least one catalytic cation comprising magnesium and/or manganese. In some embodiments, the at least one nucleotide binds the sequencing polymerase and incorporates into the 3' end of the extendible primer. In some embodiments, the incorporating the nucleotide into the 3' end of the primer in **step (c)** comprises a primer extension reaction.

**[0357]** In some embodiments, the methods for sequencing further comprise **step (d)**: repeating the incorporating at least one nucleotide into the 3' end of the extendible primer of step (c) at least once. In some embodiments, the plurality of nucleotides comprises a plurality of nucleotides labeled with detectable reporter moiety. The detectable reporter moiety comprises a fluorophore. In some embodiments, the fluorophore is attached to the nucleotide base. In some embodiments, the fluorophore is attached to the nucleotide base with a linker which is cleavable/removable from the base. In some embodiments, at least one of the nucleotides in the plurality is not labeled with a detectable reporter moiety. In some embodiments, a particular detectable reporter moiety (e.g., fluorophore) that is attached to the nucleotide can correspond to the nucleotide base (e.g., dATP, dGTP, dCTP, dTTP or dUTP) to permit detection and identification of the nucleotide base. In some embodiments, the method further comprises detecting the at least one incorporated nucleotide at step (c) and/or (d). In some embodiments, the method further comprises identifying the at least one incorporated nucleotide at step (c) and/or (d). In some embodiments, the sequence of the

nucleic acid concatemer molecule can be determined by detecting and identifying the nucleotide that binds the sequencing polymerase, thereby determining the sequence of the concatemer molecule. In some embodiments, the sequence of the nucleic acid concatemer molecule can be determined by detecting and identifying the nucleotide that incorporates into the 3' end of the primer, thereby determining the sequence of the concatemer molecule.

**[0358]** In some embodiments, in the methods for sequencing, the plurality of sequencing polymerases that are bound to the nucleic acid duplexes comprise a plurality of complexed polymerases, having at least a first and second complexed polymerase, wherein (a) the first complexed polymerases comprises a first sequencing polymerase bound to a first nucleic acid duplex comprising a first nucleic acid template sequence which is hybridized to a first nucleic acid primer, (b) the second complexed polymerases comprises a second sequencing polymerase bound to a second nucleic acid duplex comprising a second nucleic acid template sequence which is hybridized to a second nucleic acid primer, (c) the first and second nucleic acid template sequences comprise the same or different sequences, (d) the first and second nucleic acid concatemers are clonally-amplified, (e) the first and second primers comprise extendible 3' ends or non-extendible 3' ends, and (f) the plurality of complexed polymerases are immobilized to a support. In some embodiments, the density of the plurality of complexed polymerases is about  $10^2 - 10^{15}$  complexed polymerases per  $\text{mm}^2$  that are immobilized to the support.

### **Two-Stage Methods for Nucleic Acid Sequencing**

**[0359]** The present disclosure provides a two-stage method for sequencing nucleic acid molecules. In some embodiments, the first stage generally comprises binding multivalent molecules to complexed polymerases to form multivalent-complexed polymerases, and detecting the multivalent-complexed polymerases.

**[0360]** In some embodiments, the first stage comprises **step (a)**: contacting a plurality of a first sequencing polymerase to (i) a plurality of nucleic acid concatemer molecules and (ii) a plurality of nucleic acid primers, wherein the contacting is conducted under a condition suitable to bind the plurality of first sequencing polymerases to the plurality of nucleic acid concatemer molecules and the plurality of nucleic acid primers thereby forming 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 concatemer

molecule hybridized to a nucleic acid primer. In some embodiments, the first polymerase comprises a recombinant mutant sequencing polymerase.

**[0361]** In some embodiments, in the methods for sequencing concatemer molecules, the primer comprises a 3' extendible end or a 3' non-extendible end. In some embodiments, the plurality of nucleic acid concatemer molecules comprise amplified template molecules (e.g., clonally amplified template molecules). In some embodiments, the plurality of nucleic acid concatemer molecules comprise one copy of a target sequence of interest. In some embodiments, the plurality of nucleic acid molecules comprise two or more tandem copies of a target sequence of interest (e.g., concatemers). In some embodiments, the nucleic acid concatemer molecules in the plurality of nucleic acid concatemer molecules comprise the same target sequence of interest or different target sequences of interest. In some embodiments, the plurality of nucleic acid concatemer molecules and/or the plurality of nucleic acid primers are in solution or are immobilized to a support. In some embodiments, when the plurality of nucleic acid concatemer molecules and/or the plurality of nucleic acid primers are immobilized to a support, the binding with the first sequencing polymerase generates a plurality of immobilized first complexed polymerases. In some embodiments, the plurality of nucleic acid concatemer molecules and/or nucleic acid primers are immobilized to  $10^2 - 10^{15}$  different sites on a support. In some embodiments, the binding of the plurality of concatemer molecules and nucleic acid primers with the plurality of first sequencing polymerases generates a plurality of first complexed polymerases immobilized to  $10^2 - 10^{15}$  different sites on the support. In some embodiments, the plurality of immobilized first complexed polymerases on the support are immobilized to pre-determined or to random sites on the support. In some embodiments, the plurality of immobilized first complexed polymerases are in fluid communication with each other to permit flowing a solution of reagents (e.g., enzymes including sequencing polymerases, multivalent molecules, nucleotides, and/or divalent cations) onto the support so that the plurality of immobilized complexed polymerases on the support are reacted with the solution of reagents in a massively parallel manner.

**[0362]** In some embodiments, the methods for sequencing further comprise **step (b)**: contacting the plurality of first complexed polymerases with a plurality of multivalent molecules to form a plurality of multivalent-complexed polymerases (e.g., binding complexes). In some embodiments, 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 (e.g., nucleotide unit) (e.g., FIGS. 12-16). In some

embodiments, the contacting of step (b) 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. In some embodiments, the condition is suitable for inhibiting polymerase-catalyzed incorporation of the complementary nucleotide units into the primers of the plurality of multivalent-complexed polymerases. In some embodiments, the plurality of multivalent molecules comprise at least one multivalent molecule having multiple nucleotide arms (e.g., FIG. 15) each attached with a nucleotide analog (e.g., nucleotide analog unit), where the nucleotide analog includes a chain terminating moiety at the sugar 2' and/or 3' position. In some embodiments, the plurality of multivalent molecules comprises at least one multivalent molecule comprising multiple nucleotide arms each attached with a nucleotide unit that lacks a chain terminating moiety. In some embodiments, at least one of the multivalent molecules in the plurality of multivalent molecules is labeled with a detectable reporter moiety. Any portion of the multivalent molecule can be labeled including the core, nucleotide arm or nucleobase. In some embodiments, the detectable reporter moiety comprises a fluorophore. In some embodiments, the contacting of step (b) is conducted in the presence of at least one non-catalytic cation comprising strontium, barium and/or calcium.

**[0363]** In some embodiments, the methods for sequencing further comprise **step (c):** detecting the plurality of multivalent-complexed polymerases. In some embodiments, the detecting includes detecting the multivalent molecules that are bound to the complexed polymerases, where the complementary nucleotide units of the multivalent molecules are bound to the primers but incorporation of the complementary nucleotide units is inhibited. In some embodiments, the multivalent molecules are labeled with a detectable reporter moiety to permit detection. In some embodiments, the labeled multivalent molecules comprise a fluorophore attached to the core, linker and/or nucleotide unit of the multivalent molecules.

**[0364]** In some embodiments, the methods for sequencing further comprise **step (d):** identifying the base of the complementary nucleotide units that are bound to the plurality of first complexed polymerases, thereby determining the sequence of the concatemer molecule. In some embodiments, the multivalent molecules are labeled with a detectable reporter moiety that corresponds to the particular nucleotide units attached to the nucleotide arms to permit identification of the complementary nucleotide units (e.g., nucleotide base adenine, guanine, cytosine, thymine or uracil) that are bound to the plurality of first complexed polymerases.

[0365] In some embodiments, the second stage of the two-stage sequencing method generally comprises nucleotide incorporation. In some embodiments, the methods for sequencing further comprise **step (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.

[0366] In some embodiments, the methods for sequencing further comprises **step (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 nucleic acid duplex. In some embodiments, the second sequencing polymerase comprises a recombinant mutant sequencing polymerase.

[0367] In some embodiments, the plurality of first sequencing polymerases of step (a) have an amino acid sequence that is 100% identical to the amino acid sequence as the plurality of the second sequencing polymerases of step (f). In some embodiments, the plurality of first sequencing polymerases of step (a) have an amino acid sequence that differs from the amino acid sequence of the plurality of the second sequencing polymerases of step (f).

[0368] In some embodiments, the methods for sequencing further comprise **step (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 thereby forming a plurality of nucleotide-complexed polymerases. In some embodiments, the contacting of step (g) is conducted under a condition that is suitable for promoting polymerase-catalyzed incorporation of the bound complementary nucleotides into the primers of the nucleotide-complexed polymerases thereby forming a plurality of nucleotide-complexed polymerases. In some embodiments, the incorporating the nucleotide into the 3' end of the primer in step (g) comprises a primer extension reaction. In some embodiments, the contacting of step (g) is conducted in the presence of at least one catalytic cation comprising magnesium and/or manganese. In some embodiments, the contacting of step (g) is conducted in the presence of magnesium and/or manganese. In some embodiments, the plurality of nucleotides comprise native nucleotides (e.g., non-analog nucleotides) or nucleotide analogs. In some embodiments, the plurality of nucleotides comprise a 2' and/or 3' chain terminating moiety which is removable or is not removable. In some embodiments,

the plurality of nucleotides comprises a plurality of nucleotides that are non-labeled or labeled with detectable reporter moiety. The detectable reporter moiety comprises a fluorophore. In some embodiments, the fluorophore is attached to the nucleotide base. In some embodiments, the fluorophore is attached to the nucleotide base with a linker which is cleavable/removable from the base or is not removable from the base. In some embodiments, at least one of the nucleotides in the plurality is not labeled with a detectable reporter moiety. In some embodiments, a particular detectable reporter moiety (e.g., fluorophore) that is attached to the nucleotide can correspond to the nucleotide base (e.g., dATP, dGTP, dCTP, dTTP or dUTP) to permit detection and identification of the nucleotide base.

**[0369]** In some embodiments, the methods for sequencing further comprise **step (h):** detecting the complementary nucleotides which are incorporated into the primers of the nucleotide-complexed polymerases. In some embodiments, the plurality of nucleotides are labeled with a detectable reporter moiety to permit detection. In some embodiments, in the methods for sequencing concatemer molecules, the detecting step is omitted.

**[0370]** In some embodiments, the methods for sequencing further comprise **step (i):** identifying the bases of the complementary nucleotides which are incorporated into the primers of the nucleotide-complexed polymerases. In some embodiments, the identification of the incorporated complementary nucleotides in step (i) can be used to confirm the identity of the complementary nucleotides of the multivalent molecules that are bound to the plurality of first complexed polymerases in step (d). In some embodiments, the identifying of step (i) can be used to determine the sequence of the nucleic acid concatemer molecules. In some embodiments, in the methods for sequencing concatemer molecules, the identifying step is omitted.

**[0371]** In some embodiments, the methods for sequencing further comprise **step (j):** removing the chain terminating moiety from the incorporated nucleotide when step (g) is conducted by contacting the plurality of second complexed polymerases with a plurality of nucleotides that comprise at least one nucleotide having a 2' and/or 3' chain terminating moiety.

**[0372]** In some embodiments, the methods for sequencing further comprise **step (k):** repeating step (a) – (g) and (j) at least once. In some embodiments, the methods for sequencing further comprise **step (k):** repeating steps (a) – (j) at least once. In some embodiments, the sequence of the nucleic acid concatemer molecules can be determined by detecting and identifying the multivalent molecules that bind the sequencing polymerases but do not incorporate into the 3' end of the primer at steps (c) and (d). In some embodiments, the

sequence of the nucleic acid concatemer molecule can be determined (or confirmed) by detecting and identifying the nucleotide that incorporates into the 3' end of the primer at steps (h) and (i).

**[0373]** In some embodiments, in any of the methods for sequencing nucleic acid molecules, the binding of the plurality of first complexed polymerases with the plurality of multivalent molecules forms at least one avidity complex, the method comprising the steps: **(a)** binding a first nucleic acid primer, a first sequencing polymerase, and a first multivalent molecule to a first portion of a concatemer template molecule thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first sequencing polymerase; and **(b)** binding a second nucleic acid primer, a second sequencing polymerase, and the first multivalent molecule to a second portion of the same concatemer template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the first 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. In some embodiments, the first sequencing polymerase comprises any wild type or mutant polymerase described herein. In some embodiments, the second sequencing polymerase comprises any wild type or mutant polymerase described herein. The concatemer template molecule comprises tandem repeat sequences of a sequence of interest and at least one universal sequencing primer binding site. The first and second nucleic acid primers can bind to a sequencing primer binding site along the concatemer template molecule. Exemplary multivalent molecules are shown in FIGS. 12-15.

**[0374]** In some embodiments, in any of the methods for sequencing nucleic acid molecules, wherein the method includes binding the plurality of first complexed polymerases with the plurality of multivalent molecules to form at least one avidity complex, the method comprising the steps: **(a)** contacting the plurality of sequencing polymerases and the plurality of nucleic acid primers with different portions of a concatemer nucleic acid concatemer molecule to form at least first and second complexed polymerases on the same concatemer template molecule; **(b)** contacting a plurality of detectably labeled multivalent molecules to the at least first and second complexed polymerases on the same concatemer 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 primer hybridized to a first portion of the concatemer template molecule thereby forming a first binding complex (e.g., first ternary 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 primer hybridized to a second portion of the concatemer template molecule thereby forming a second binding complex (e.g., second ternary 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 concatemer template molecule, and **(d)** identifying the first nucleotide unit in the first binding complex thereby determining the sequence of the first portion of the concatemer template molecule, and identifying the second nucleotide unit in the second binding complex thereby determining the sequence of the second portion of the concatemer template molecule. In some embodiments, the plurality of sequencing polymerases comprise any wild type or mutant sequencing polymerase described herein. The concatemer template molecule comprises tandem repeat sequences of a sequence of interest and at least one universal sequencing primer binding site. The plurality of nucleic acid primers can bind to a sequencing primer binding site along the concatemer template molecule. Exemplary multivalent molecules are shown in FIGS. 12-15.

### Sequencing-by-Binding

**[0375]** The present disclosure provides methods for sequencing any of the immobilized concatemer molecules described herein, wherein the sequencing methods comprise a sequencing-by-binding (SBB) procedure which employs non-labeled chain-terminating nucleotides. In some embodiments, the sequencing-by-binding (SBB) method comprises the steps of **(a)** sequentially contacting a primed template nucleic acid with at least two separate mixtures under ternary complex stabilizing conditions, wherein the at least two separate mixtures each include a polymerase and a nucleotide, whereby the sequentially contacting results in the primed template nucleic acid being contacted, under the ternary complex stabilizing conditions, with nucleotide cognates for first, second and third base type base types in the template; **(b)** examining the at least two separate mixtures to determine whether a ternary complex formed; and **(c)** identifying the next correct nucleotide for the primed template nucleic acid molecule, wherein the next correct nucleotide is identified as a cognate of the first, second or third base type if ternary complex is detected in step (b), and wherein the next correct nucleotide is imputed to be a nucleotide cognate of a fourth base type based on the absence of a ternary complex in step (b); **(d)** adding a next correct nucleotide to the

primer of the primed template nucleic acid after step (b), thereby producing an extended primer; and **(e)** repeating steps (a) through (d) at least once on the primed template nucleic acid that comprises the extended primer. Exemplary sequencing-by-binding methods are described in U.S. patent Nos. 10,246,744 and 10,731,141 (where the contents of both patents are hereby incorporated by reference in their entireties).

### **Multiplex Workflows**

**[0376]** The present disclosure provides multiplex workflows which generally comprise preparing separate populations of sample-indexed covalently closed circular library molecules using double-stranded splint adaptors (200) and sample-indexed nucleic acid libraries carrying one or both index sequences (e.g., left (160) and/or right (170) sample index sequences). The first left index sequence (160) and the first right index sequence (170) are known sequences. Separate sample-indexed libraries can be prepared from input nucleic acids isolated from different sources where the sample index sequences are used to distinguish the different sources. The pooling step can be conducted either after generating sample-indexed covalently closed circular library molecules or before generating sample-indexed library-splint complexes. The pooled molecules can be subjected to downstream multiplex amplification and/or multiplex sequencing reactions.

### **A Conventional Pooling Workflow for Multiplexing**

**[0377]** In one embodiment, a method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources, comprises: **(a)** providing two or more populations of single-stranded nucleic acid library molecules (100), each population of library molecules (100) contained in a separate compartment, wherein the nucleic acid library molecules in a given population comprise **(i)** a first left universal adaptor sequence (120) having a binding sequence for a first surface primer, **(ii)** a second left universal adaptor sequence (140) having a binding sequence for a first sequencing primer, **(iii)** a sequence of interest (110) isolated from a sample source, **(iv)** a second right universal adaptor sequence (150) having a binding sequence for a second sequencing primer, **(v)** a first right universal adaptor sequence (130) having a binding sequence for a second surface primer, and **(vii)** a first left index sequence (160) and/or a first right index sequence (170), wherein the sequences of the left (160) and/or right (170) indexes separately or in combination identify the sample source from which the sequences of interest (110) have been isolated (e.g., see

FIGS. 5-7). In some embodiments, the single-stranded nucleic acid library molecules (100) further comprises a first left unique identification sequence (180) and/or a first right unique identification sequence (190).

**[0378]** In some embodiments, the method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources further comprises step **(b)**: providing a plurality of double-stranded splint adaptors (200) wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the first splint strand (300) comprises regions arranged in a 5' to 3' order a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the second splint strand comprises regions arranged in a 5' to 3' order **(i)** a second sub-region having a universal binding sequence for a fourth surface primer, and **(ii)** a first sub-region having a universal binding sequence for a third surface primer.

**[0379]** In some embodiments, the method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources further comprises step **(c)**: contacting in the separate compartments the population of single-stranded nucleic acid library molecules (100) with an allotment of the plurality of double-stranded splint adaptors (200), wherein the contacting is conducted under a condition suitable to hybridize portions of the first splint strand (300) to portions of the library molecules (100) thereby circularizing the library molecules to generate a population of library-splint complexes (500), such that the first region (320) of an individual first splint strand is hybridized to the binding sequence for the first surface primer (120) of an individual library molecule (100), and the third region (330) of the individual first splint strand is hybridized to the binding sequence for the second surface primer (130) of the individual library molecule (100), wherein each of the library-splint complexes (500) comprise a first nick between the 5' end of the library molecule and the 3' end of the second splint strand (300), wherein each of the library-splint complexes (500) comprises a second nick between the 5' end of the second splint strand (300) and the 3' end of the library molecule (100), and wherein the first and second nicks are enzymatically ligatable (e.g., see FIGS. 5-7).

**[0380]** In some embodiments, the method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources further comprises step **(d)**: contacting in the separate compartments the populations of library-splint complexes (500) with a ligase, under a condition suitable to enzymatically ligate the first and second nicks, thereby

generating a population of covalently closed circular library molecules (600) each hybridized to the first splint strand (300) (e.g., see FIGS. 7 and 8).

**[0381]** In some embodiments, the method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources further comprises step (e): pooling together the population of covalently closed circular library molecules (600) from the separate compartments to generate a multiplex mixture of covalently closed circular library molecules (600) which comprise the multiplex mixture of sequences-of-interest isolated from a plurality of sample sources.

**[0382]** In some embodiments, the sequences of interest can be isolated from two or more different sample sources (e.g., 2-10, or 10-50, or 50-100, or 100-250, or more than 250 different sample sources). Exemplary nucleic acid sources include naturally-occurring, recombinant, or chemically-synthesized sources. Exemplary nucleic acid sources include single cells, a plurality of cells, tissue, biological fluid, environmental sample or whole organism. Exemplary nucleic acid sources include fresh, frozen, fresh-frozen or archived sources (e.g., formalin-fixed paraffin-embedded; FFPE). The skilled artisan will recognize that the nucleic acids can be isolated from many other sources. The sequences of interest in a given population have the same or different sequences.

**[0383]** In some embodiments, the number of populations of single-stranded nucleic acid library molecules (100) of step (a) can be 2-10, or 10-50, or 50-100, or 100-250, or more than 250 different populations of single-stranded nucleic acid library molecules (100). In some embodiments, any number of different populations of covalently closed circular library molecules (600) can be pooled together in step (e), for example 2-10, or 10-50, or 50-100, or 100-200, or more than 200 different populations of covalently closed circular library molecules (600) can be pooled together.

**[0384]** The skilled artisan will recognize that any number of separate compartments can be used in step (a) (e.g., 2-10, or 10-50, or 50-100, or 100-250, or more separate compartments) (e.g., multi-well plate such as for example a 96-well plate).

**[0385]** In some embodiments, the 3' end of the first splint strand (300) that are hybridized to the covalently closed circular library molecules (600) of step (d) or (e) comprise an extendible 3'OH ends which can serve as an initiation point for a primer extension reaction (e.g., rolling circle amplification reaction).

**[0386]** In some embodiments, at step (d) or (e) the population of covalently closed circular library molecules (600) that are hybridized to the first splint strand (300) can optionally be reacted with at least one exonuclease enzyme to remove the plurality of first splint strands

(300) and retaining the plurality of covalently closed circular library molecules (600). In some embodiments, the at least one exonuclease enzyme comprises exonuclease I, thermolabile exonuclease I and/or T7 exonuclease.

**[0387]** In some embodiments, the single-stranded nucleic acid library molecules (100) of step (a) further comprise any one or any combination of two or more of: a universal binding sequence for a forward amplification primer; a universal binding sequence for a reverse amplification primer; and/or a universal binding sequence for a compaction oligonucleotide.

**[0388]** In some embodiments, individual covalently closed circular library molecules (600) in the multiplex mixture of step (e) comprise a second splint strand region (400) which includes a universal binding sequence for a third surface primer.

### **A Non-Conventional Pooling Workflow for Multiplexing**

**[0389]** In one embodiment, a method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources, comprises: **(a)** providing two or more populations of single-stranded nucleic acid library molecules (100), each population of library molecules (100), each population of single-stranded nucleic acid library molecules is contained in a separate compartment, wherein the nucleic acid library molecules in a given population comprise **(i)** a first left universal adaptor sequence (120) having a binding sequence for a first surface primer, **(ii)** a second left universal adaptor sequence (140) having a binding sequence for a first sequencing primer, **(iii)** a sequence of interest (110) isolated from a sample source, **(iv)** a second right universal adaptor sequence (150) having a binding sequence for a second sequencing primer, **(v)** a first right universal adaptor sequence (130) having a binding sequence for a second surface primer, and **(vii)** a first left index sequence (160) and/or a first right index sequence (170), wherein the sequences of the left (160) and/or right (170) indexes separately or in combination identify the sample source from which the sequences of interest (110) are isolated (e.g., see FIGS. 5-7). In some embodiments, the single-stranded nucleic acid library molecules (100) further comprises a first left unique identification sequence (180) and/or a first right unique identification sequence (190).

**[0390]** In some embodiments, the method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources further comprises step **(b)**: pooling together the two or more populations of single-stranded nucleic acid library molecules (100) into the same compartment.

**[0391]** In some embodiments, the method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources further comprises step **(c)**: adding to

the compartment of step (b) a plurality of double-stranded splint adaptors (200) wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the first splint strand (300) comprises regions arranged in a 5' to 3' order a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the second splint strand comprises regions arranged in a 5' to 3' order (i) a second sub-region having a universal binding sequence for a fourth surface primer, and (ii) a first sub-region having a universal binding sequence for a third surface primer.

**[0392]** In some embodiments, the method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources further comprises step (d): incubating the population of single-stranded nucleic acid library molecules (100) and the plurality of double-stranded splint adaptors (200) which are in the same compartment of step (c) under a condition suitable to hybridize portions of the first splint strand (300) to portions of the library molecules (100) thereby circularizing the library molecules to generate a population of library-splint complexes (500), such that the first region (320) of an individual first splint strand is hybridized to the binding sequence for the first surface primer (120) of an individual library molecule (100), and the third region (330) of the individual first splint strand is hybridized to the binding sequence for the second surface primer (130) of the individual library molecule (100), wherein each of the library-splint complexes (500) comprise a first nick between the 5' end of the library molecule and the 3' end of the second splint strand (300), wherein each of the library-splint complexes (500) comprises a second nick between the 5' end of the second splint strand (300) and the 3' end of the library molecule (100), and wherein the first and second nicks are enzymatically ligatable.

**[0393]** In some embodiments, the method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources further comprises step (e): contacting the populations of library-splint complexes (500) in the compartment of step (d) with a ligase, under a condition suitable to enzymatically ligate the first and second nicks, thereby generating a population of covalently closed circular library molecules (600) each hybridized to the first splint strand (300), thereby generating a multiplex mixture of covalently closed circular library molecules (600) which comprise the multiplex mixture of sequences-of-interest isolated from a plurality of sample sources (e.g., see FIGS. 7 and 8).

**[0394]** In some embodiments, the sequences of interest can be isolated from two or more different sample sources (e.g., 2-10, or 10-50, or 50-100, or 100-250, or more than 250

different sample sources). Exemplary nucleic acid sources include naturally-occurring, recombinant, or chemically-synthesized sources. Exemplary nucleic acid sources include single cells, a plurality of cells, tissue, biological fluid, environmental sample or whole organism. Exemplary nucleic acid sources include fresh, frozen, fresh-frozen or archived sources (e.g., formalin-fixed paraffin-embedded; FFPE). The skilled artisan will recognize that the nucleic acids can be isolated from many other sources. The sequences of interest in a given population have the same or different sequences.

**[0395]** In some embodiments, the number of populations of single-stranded nucleic acid library molecules (100) of step (a) can be 2-10, or 10-50, or 50-100, or 100-250, or more than 250 different populations of single-stranded nucleic acid library molecules (100). In some embodiments, any number of different populations of single-stranded nucleic acid library molecules can be pooled together in step (b), for example 2-10, or 10-50, or 50-100, or 100-200, or more than 200 different populations of covalently closed circular library molecules (600) can be pooled together.

**[0396]** The skilled artisan will recognize that any number of separate compartments can be used in step (a) (e.g., 2-10, or 10-50, or 50-100, or 100-250, or more separate compartments) (e.g., multi-well plate such as for example a 96-well plate).

**[0397]** In some embodiments, the 3' ends of the first splint strand (300) that are hybridized to the covalently closed circular library molecules (600) of step (e) comprise extendible 3'OH ends which can serve as an initiation point for a primer extension reaction (e.g., rolling circle amplification reaction).

**[0398]** In some embodiments, at step (e) the population of covalently closed circular library molecules (600) that are hybridized to the first splint strand (300) can optionally be reacted with at least one exonuclease enzyme to remove the plurality of first splint strands (300) and retaining the plurality of covalently closed circular library molecules (600). In some embodiments, the at least one exonuclease enzyme comprises exonuclease I, thermolabile exonuclease I and/or T7 exonuclease.

**[0399]** In some embodiments, the single-stranded nucleic acid library molecules (100) of step (a) further comprise any one or any combination of two or more of: a universal binding sequence for a forward amplification primer; a universal binding sequence for a reverse amplification primer; and/or a universal binding sequence for a compaction oligonucleotide.

**[0400]** In some embodiments, individual covalently closed circular library molecules (600) in the multiplex mixture of step (e) comprise a second splint strand region (400) which includes a universal binding sequence for a third surface primer.

**On-Support Multiplex Rolling Circle Amplification Reaction**

**[0401]** In some embodiments, any of the multivalent mixtures of covalently closed circular library molecules (600) described herein can be distributed onto a support, wherein the method comprises: distributing the plurality of covalently closed circular library molecules (600) onto a support having a plurality of the fourth surface primers immobilized on the support, under a condition suitable for hybridizing individual covalently closed circular library molecules (600) to individual immobilized fourth surface primers thereby immobilizing the plurality of covalently closed circular library molecules (600); and contacting the plurality of immobilized covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of fourth surface primers as immobilized amplification primers and the plurality of covalently closed circular library molecules (600) as template molecules, thereby generating a plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP. In some embodiments, the population of covalently closed circular library molecules are still hybridized to the first splint strand (300) and are distributed onto the support. In some embodiments, the first splint strands have been removed from the population of covalently closed circular library molecules (600) using at least one exonuclease enzyme under a condition suitable to remove the plurality of first splint strands (300) and retain the population of covalently closed circular library molecules (600).

**In-Solution Multiplex Rolling Circle Amplification Reaction using Soluble Amplification Primers and Immobilizing on a Support**

**[0402]** In some embodiments, any of the multivalent mixtures of covalently closed circular library molecules (600) described herein can be subjected to an in-solution rolling circle amplification reaction, the method comprising: contacting the population of covalently closed circular library molecules (600) that are hybridized to the first splint strand (300) with at least one exonuclease enzyme under a condition suitable to remove the plurality of first splint strands (300) and retaining the population of covalently closed circular library molecules (600); and contacting the retained population of covalently closed circular library molecules (600) with a plurality of soluble amplification primers, a plurality of strand-displacing polymerases, and a plurality of nucleotides, under a condition suitable to conduct a rolling

circle amplification reaction thereby generating a plurality of nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP. In some embodiments, the covalently closed circular library molecules (600) comprise a universal binding sequence for a forward amplification primer and/or a universal binding sequence for a reverse amplification primer. In some embodiments, the soluble amplification primers can hybridize to the universal binding sequence for a forward amplification primer or the universal binding sequence for a reverse amplification primer. In some embodiments, the at least one exonuclease enzyme comprises exonuclease I, thermolabile exonuclease I and/or T7 exonuclease.

**[0403]** In some embodiments, the plurality of nucleic acid concatemer molecules can be distributed onto a support, the method comprising: distributing the plurality of concatemer molecules onto a support having a plurality of the fourth surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized fourth surface primers thereby immobilizing the plurality of concatemer molecules; and contacting the immobilized plurality of concatemer molecules (which are hybridized to a covalently closed circular library molecule (600)) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

#### **In-Solution Multiplex Rolling Circle Amplification Reaction using First Splint Strands and Immobilizing on a Support**

**[0404]** In some embodiments, any of the multivalent mixtures of covalently closed circular library molecules (600) described herein can be subjected to an in-solution rolling circle amplification reaction, the method comprising: conducting a rolling circle amplification reaction by contacting the population of covalently closed circular library molecules (600) which are hybridized to the first splint strand (300) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction thereby generating a plurality of concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0405]** In some embodiments, the plurality of concatemer molecules can be distributed onto a support, the method comprising: distributing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600) onto a support having a plurality of the fourth surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized fourth surface primers thereby immobilizing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600); and contacting the plurality of immobilized concatemer molecules with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

#### **Multiplex Sequencing the Immobilized Concatemer Molecules**

**[0406]** Any of the multiplex immobilized concatemer molecules described herein can be subjected to sequencing reactions, which generally comprise: sequencing the insert region (110) and the first left index sequence (160) and/or the first right index sequence (170) of the plurality of immobilized nucleic acid concatemer molecules, wherein the sequences of the insert region (110) along with the first left index sequence (160) and/or first right index sequence (170) can be used to identify the sample source of the sequence of interest of a given immobilized concatemer molecule. The immobilized nucleic acid concatemer molecules comprises the insert region (110) and one or both of the sample index sequences (e.g., first left index sequence (160) and/or the first right index sequence (170)). The insert region (110), the first left index sequence (160) and/or the first right index sequence (170) can be sequenced in any order. In some embodiments, the insert region (110) can be sequenced first, the first left index sequence (160) can be sequenced second, and the first right index sequence (170) can be sequenced third if it is present in the immobilized nucleic acid concatemer molecule. In some embodiments, the first left index sequence (160) can be sequenced first, the insert region (110) can be sequenced second, and the first right index sequence (170) can be sequenced third if it is present in the immobilized nucleic acid concatemer molecule.

**[0407]** In some embodiments, the sequencing reads of the first left index (160) and the first right index (170) can be aligned with their respective known sequences and assigned an

alignment score, where the alignment score indicates the similarity between the known index sequence and the sequencing read of that index. The alignment score(s) of the first left index (160) and/or the first right index (170), along with the sequence read of the associated insert region (110), can be used to determine the sample source of the insert region.

### **Multiplex Sequencing using Nucleotide Analogs**

**[0408]** In some embodiments, any of the multiplex immobilized concatemer molecules described herein can be sequenced, the sequencing method comprising: (a) binding the plurality of immobilized nucleic acid concatemer molecules with a plurality of sequencing primers, a plurality of sequencing polymerases, and a plurality of detectably labeled nucleotide analogs each comprising a 2' or 3' chain terminating moiety, under a condition suitable for incorporating the detectably labeled nucleotide analogs into the 3' end of the sequencing primers; (b) detecting the incorporated detectably labeled nucleotide analog; and (c) identifying the nucleobase of the incorporated detectably labeled nucleotide analog. Sequencing methods that employ nucleotide analogs are described in detail above.

### **Multiplex Sequencing using a Two-Stage Sequencing Method**

**[0409]** In some embodiments, any of the multiplex immobilized concatemer molecules described herein can be sequenced using a two-stage sequencing method, wherein the first stage comprises: **(a)** binding the plurality of immobilized nucleic acid concatemer molecules with a plurality of sequencing primers, a plurality of a first sequencing polymerase, and a plurality of detectably labeled multivalent molecules, under a condition suitable to bind individual concatemer molecules to a sequencing primer, a first sequencing polymerase, and a detectably labeled multivalent molecule (e.g., forming a plurality of multivalent-complexed polymerases), where the suitable conditions inhibit polymerase-catalyzed incorporation of the nucleotide unit of the detectably labeled multivalent molecule; **(b)** detecting the bound detectably labeled multivalent molecule; and **(c)** identifying the nucleobase of the detectably labeled multivalent molecule. In some embodiments, individual detectably labeled multivalent molecules comprise a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide (e.g., nucleotide unit) (e.g., see FIGS. 12-16). In some embodiments, at least one of the multivalent molecules in the plurality of multivalent molecules is labeled with a detectable reporter moiety. Any portion of the multivalent molecule can be labeled including the core, nucleotide arm or nucleobase. In some embodiments, the detectable reporter moiety comprises a fluorophore. In some embodiments,

the binding of step (a) is conducted in the presence of at least one non-catalytic cation comprising strontium, barium and/or calcium. In some embodiments, the binding of the detectably labeled multivalent molecules to the plurality of immobilized nucleic acid concatemer molecules forms at least one avidity complex as described above.

**[0410]** In some embodiments, the second stage of the two-stage sequencing method comprises: **(d)** removing the plurality of first sequencing polymerases and the plurality of bound multivalent molecules from the plurality of immobilized nucleic acid concatemer molecules and retaining the immobilized concatemer molecules each hybridized to a sequencing primer (nucleic acid duplex); and **(e)** contacting the retained immobilized concatemer molecules and hybridized sequencing primers with a plurality of second polymerases and a plurality of nucleotides under a condition suitable for incorporating the nucleotides into the 3' end of the sequencing primers. Sequencing methods that employ the two-stage methods are described in detail above.

### **Sample Indexes for Improved Base Calling**

**[0411]** Generally, it is desirable to prepare nucleic acid libraries that will be distributed onto a support (e.g., coated flowcell), where the library molecules are converted into template molecules that are immobilized at a high density to the support for massively parallel sequencing. For template molecules that are immobilized at high densities at random locations on the support, the challenge of resolving high density fluorescent images for accurate base calling during sequencing runs becomes challenging.

**[0412]** The nucleotide diversity of a population of immobilized template molecules refers to the relative proportion of nucleotides A, G, C and T that are present in each sequencing cycle. An optimal high diversity library will generally include sequence-of-interest (insert) regions having approximately equal proportions of all four nucleotides represented in each cycle of a sequencing run. A low diversity library will generally include sequence-of-interest (insert) regions having a high proportion of certain nucleotides and low proportion of other nucleotides. To overcome the problem of low diversity libraries, a small amount of a high diversity library prepared from PhiX bacteriophage is typically mixed with the library-of-interest (e.g., PhiX spike-in library) and sequenced together on the same flowcell. While the PhiX library spike-in library provides nucleotide diversity it also occupies space on the flowcell thereby replacing the target libraries carrying the sequence-of-interest and reduces the amount of sequencing data obtainable from the target libraries (e.g., reduces sequencing

throughput). Another method to overcome the problem of low diversity libraries is to prepare target library molecules having at least one sample index sequence that is designed to be color-balanced. However it may be desirable to design a large number of sample index sets, for example a set of single index sample sequences or paired index sample sequences for 16-plex, 24-plex, 96-plex or larger plexy levels. It is challenging to design sample index sequences, as a single or paired sample indexes, for large sample index sets where all of the sample index sequences are color-balanced (e.g., see FIG. 26).

**[0413]** An alternative method to overcome the challenges of sequencing low diversity library molecules (e.g., at high density on the support) is to prepare libraries having at least one sample index sequence comprising a short random sequence (e.g., NNN) linked directly to a universal sample index sequence, where the short random sequence provides nucleotide diversity and color balance. Table 1, at FIGS. 23A-23F, provides a list of exemplary right index sequences (170) having a short random sequence (e.g., NNN) and a universal sample index sequence. In a population of sample-indexed library molecules, the short random sequence of the sample index (e.g., (170)) provides high nucleotide diversity which includes approximately equal proportions of all four nucleotides (e.g., A, G, C, T and/or U) that will be represented in each cycle of a sequencing run (see FIG. 26). The high nucleotide diversity of the short random sequence also provide color balance during each cycle of the sequencing run. The advantage of designing sample indexes (e.g., (160) and/or (170)) to include a short random sequence (e.g., NNN) is that, in a low-plexy population of library molecules (e.g., 2-plex or 4-plex), the universal sample index sequences that identify the two or four different samples need not exhibit nucleotide diversity (e.g., see FIG. 27). Additionally, the nucleotide diversity of the short random sequence (e.g., NNN) can obviate the need to include a PhiX spike-in library, or permits use of a reduced amount of PhiX spike-in library to be distributed onto the flowcell and sequenced.

**[0414]** The target library molecule can include a single sample index sequence which includes a short random sequence (e.g., sample index (170)) and a universal sample index sequence. In some embodiments, the sequencing data from only the single sample index sequence (e.g., (170)) is used for polony mapping and template registration because the short random sequence (e.g., NNN) provides sufficient nucleotide diversity and color balance. The sequencing data from the universal sample index sequence (e.g., (170)) can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay.

**[0415]** The target library molecule can further include a second sample index sequence (e.g., dual sample index) comprising a second universal sample index sequence (e.g., (160)).

In some embodiments, the sequencing data from only the single sample index sequence (e.g., (170)) is used for polony mapping and/or template registration because the short random sequence provides sufficient nucleotide diversity and color balance. The sequencing data from the first universal sample index sequence (170) and the second universal sample index sequence (160) can be used as dual sample indexes to distinguish sequences of interest obtained from different sample sources in a multiplex assay. In some embodiments, the second sample index sequence (e.g., (160)) may or may not include a second short random sequence (e.g., NNN).

**[0416]** The order of sequencing the sequence-of-interest region and the sample index region(s) can also be used to improve the challenges of sequencing low diversity library molecules. For example, the sample index region can be sequenced first before sequencing the sequence-of-interest region, and the sample index sequence can be associated with the sequence-of-interest region. For example, the sample index region can be sequenced first including sequencing the short random sequence (e.g., NNN) and optionally sequencing at least a portion of the universal sample index), and then sequencing the sequence-of-interest region. In a population of sample indexed library molecules, the short random sequence (e.g., NNN) provides nucleotide diversity which may not be provided the sequence-of-interest regions of the library molecules. The sequence of the sample index provides improved nucleotide diversity and color balance for polony mapping and template registration.

**[0417]** Additionally, when sequencing the sample index region first, the length of the sequenced sample index region is relatively short (e.g., less than 30 nucleotides in length) so that de-hybridization of the product of the sequenced sample index region is more complete. Gentler de-hybridization conditions can be used to remove most or all of the product of the sequenced sample index region which reduces the level of residual signals from any sequencing products remaining hybridized to the template molecules. By contrast, the sequence-of-interest region is typically much longer than the sample index region (e.g., more than 100 nucleotides in length). When the sequence-of-interest region is sequenced before the sample index region, the product of the sequenced sequence-of-interest region must be subjected to harsher de-hybridization conditions to remove any products remaining hybridized to the template molecules which may damage the template molecules.

**[0418]** The present disclosure provides nucleic acid library molecules (100) each comprising at least one sample index sequence that can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay, where the at least one sample index sequence comprises a short random sequence (e.g., NNN) linked to a universal

sample index sequence. In some embodiments, the left sample index (160) comprises a short random sequence (e.g., NNN) linked to a universal left sample index sequence and/or the right sample index (170) comprises a short random sequence (e.g., NNN) linked to a right universal sample index sequence. The at least one sample index sequence can include sequence diversity for improved base calling. The at least one sample index sequence can be used to improve base calling accuracy.

**[0419]** In some embodiments, the short random sequence (e.g., NNN) is positioned upstream of the universal sample index sequence (e.g., (170) and/or (160)) so that during a sequencing run the random sequence portion is sequenced before the universal sample index sequence. In some embodiments, the short random sequence is positioned downstream of the universal sample index sequence so that during a sequencing run the random portion is sequenced after the universal sample index sequence.

**[0420]** In some embodiments, in the random sequence each base “N” at a given position is independently selected from A, G, C, T or U. In some embodiments, the random sequence lacks consecutive repeat sequences having 2 or 3 of the same nucleo-base, for example AA, TT, CC, GG, UU, AAA, TTT, CCC, GGG or UUU. In some embodiments, in a population of library molecules the universal sample index sequences (e.g., (170) and/or (160)) include a short random sequence having a high diversity sequence which includes approximately equal proportions of all four nucleotides (e.g., A, G, C, T and/or U) that will be represented in each cycle of a sequencing run.

**[0421]** In some embodiments, the short random sequence (e.g., NNN) comprises 3-20 nucleotides, or 3-10 nucleotides, or 3-8 nucleotides, or 3-6 nucleotides, or 3-5 nucleotides, or 3-4 nucleotides. In some embodiments, the short random sequence (e.g., NNN) comprises 3 nucleotides.

**[0422]** In some embodiments, the short random sequence (e.g., NNN) includes, but is not limited to, AGC, AGT, GAC, GAT, CAT, CAG, TAG, TAC. The skilled artisan will recognize that many more random sequences can be prepared (e.g., 64 possible combinations) where each base “N” at a given position in the random sequence is independently selected from A, G, C, T or U.

**[0423]** In some embodiments, the universal sample index sequence comprises 5-20 nucleotides, or 7-18 nucleotides, or 9-16 nucleotides.

**[0424]** In some embodiments, individual right sample index sequences (e.g., (170)) in a population of right sample index sequences comprise a universal sample index sequence and a short random sequence (e.g., NNN). In some embodiments, the short random sequences in

the population of right sample index sequences have an overall base composition of about 25% or about 20-30% of all four nucleotide bases (e.g., A, G, C and T/U) to provide nucleotide diversity at each sequencing cycle during sequencing the short random sequence (e.g., NNN).

**[0425]** In some embodiments, in the population of right sample index sequences the proportion of adenine (A) at any given position in the short random sequence is about 20-30% or about 15-35% or about 10-40%. In some embodiments, in the population of right sample index sequences the proportion of guanine (G) at any given position in the short random sequence is about 20-30% or about 15-35% or about 10-40%. In some embodiments, in the population of right sample index sequences the proportion of cytosine (C) at any given position in the short random sequence is about 20-30% or about 15-35% or about 10-40%. In some embodiments, in the population of right sample index sequences the proportion of thymine (T) or uracil (U) at any given position in the short random sequence is about 20-30% or about 15-35% or about 10-40%.

**[0426]** In some embodiments, in the population of right sample index sequences the proportion of adenine (A) and thymine (T), or the proportion of adenine (A) and uracil (U), at any given position in the short random sequence is about 10-65%. In some embodiments, in the population of right sample index sequences the proportion of guanine (G) and cytosine (C) at any given position in the short random sequence is about 10-65%.

**[0427]** In some embodiments, in the population of right sample index sequences the sequence diversity of the short random sequences ensures that no sequencing cycle is presented with fewer than four different nucleotide bases during sequencing at least the short random sequence (e.g., NNN).

**[0428]** Exemplary sample index sequences that include a short random sequence NNN linked directly to a universal sample index sequence include but are not limited to: NNNGTAGGAGCC (SEQ ID NO:97); NNNCCGCTGCTA (SEQ ID NO:98); NNNAACAACAAG (SEQ ID NO:99); NNNGGTGGTCTA (SEQ ID NO:100); NNNTTGGCCAAC (SEQ ID NO:101); NNNCAGGAGTGC (SEQ ID NO:105); and NNNATCACACTA (SEQ ID NO:106). The skilled artisan will recognize that the universal sample index can be any length and have any sequence that can be used to distinguish sequences of interest obtained from different sample sources in a multiplex assay. In a population of a given sample index, for example NNNGTAGGAGCC (SEQ ID NO:97), the population contains a mixture of individual sample index molecules each carrying the same universal sample index sequence (e.g., GTAGGAGCC) and a different short random

sequence (e.g., NNN) where up to 64 different short random sequences may be present in the population of the given sample index.

**[0429]** Exemplary sample index sequence that include a short random sequence NNN are listed in Table 1 at FIGS. 23A-23F. In some embodiments, at least one library molecule comprises a right sample index comprising a short random sequence (e.g., NNN) directly linked to a right universal sample index sequence. In some embodiments, the at least one library molecule further comprises a left sample index comprising a left universal sample index sequence that differs from the right universal sample index sequence.

**[0430]** In some embodiments, the random sequence (e.g., NNN) provides a balanced ratio of nucleobases adenine, cytosine, guanine, thymine and/or uracil (see FIG. 26). In some embodiments, in a population of sample-indexed library molecules, the random sequence (e.g., NNN) together with at least a portion of the universal sample index sequence provide a balanced ratio of nucleobases adenine, cytosine, guanine, thymine and/or uracil represented in each cycle of a sequencing run.

**[0431]** In some embodiments, a sequencing reaction includes use of polymerases and nucleotides (e.g., nucleotide analogs) that are labeled with a different fluorophore that corresponds to the nucleobase. In some embodiments, sequencing the random sequence (e.g., NNN) using labeled nucleotides provides a balanced ratio of fluorescent colors that correspond to the nucleobases adenine, cytosine, guanine, thymine and/or uracil in each cycle of a sequencing run. In some embodiments, sequencing the random sequence (e.g., NNN) and at least a portion of the universal sample index sequence using labeled nucleotides provides a balanced ratio of fluorescent colors that correspond to nucleobases adenine, cytosine, guanine, thymine and/or uracil (e.g., see FIG. 26). The labeled nucleotides emit fluorescent signals during the sequencing reactions. In some embodiments, the sequencing reaction is conducted on a sequencing apparatus having a detector that captures fluorescent images from sequencing reactions on the immobilized template molecules. The sequencing apparatus can be configured to relay the fluorescent imaging data captured by the detector to a computer system that is programmed to determine the location (e.g., mapping) of the immobilized template molecules on the flowcell. The computer system can generate a map of the locations of the immobilized template molecules based on the fluorescent imaging data of only the random sequence (e.g., NNN), or based on the random sequence (e.g., NNN) and at least a portion of the universal sample index sequence. Thus the few numbers of sequencing cycles used to sequence the random sequence (e.g., NNN) and optionally a portion of the universal sample index sequence can be used to generate a map of the location of the

immobilized template molecules. The computer system can be configured to extract the fluorescent color and intensity of only the random sequence (e.g., NNN), or the random sequence (e.g., NNN) and at least a portion of the universal sample index sequence. The computer system can be configured to use the location of a given immobilized template molecule and the fluorescent color and intensity associated with the given template molecule (which were established while sequencing the random sequence) for base calling while sequencing the insert region (110). The computer system can be configured to detect phasing and pre-phasing while sequencing the random sequence (e.g., NNN) and the universal sample index sequence, and the insert region (110). In some embodiments, the balanced ratio of fluorescent colors provided by the random sequence (e.g., NNN) at each sequencing cycle can improve the quality of the data which is processed from the fluorescent images captured by the detector, and can in turn improve the capability by the computer system to determine the location of the immobilized template molecules on the flowcell, and the color and intensity, all of which can improve base calling accuracy and quality scores of the sequenced insert region (110).

**[0432]** In some embodiments, a sequencing reaction includes use of polymerases and multivalent molecules that are labeled with a different fluorophore that corresponds to the nucleo-base (e.g., adenine, guanine, cytosine, thymine or uracil) of the nucleotide units that are attached to the nucleotide arms in a given multivalent molecule. In some embodiments, the core of individual multivalent molecules is attached to a fluorophore which corresponds to the nucleotide units (e.g., adenine, guanine, cytosine, thymine or uracil) that are attached to the nucleotide arms in a given multivalent molecule (e.g., see FIG. 26). In some embodiments, at least one of the nucleotide arms of the multivalent molecule comprises a linker and/or nucleotide base that is attached to a fluorophore, and wherein the fluorophore which is attached to a given linker or nucleotide base corresponds to the nucleotide base (e.g., adenine, guanine, cytosine, thymine or uracil) of the nucleotide arm. In some embodiments, sequencing the random sequence (e.g., NNN) using labeled multivalent molecules provides a balanced ratio of fluorescent colors that correspond to the nucleo-bases adenine, cytosine, guanine, thymine and/or uracil in each cycle of a sequencing run. In some embodiments, sequencing the random sequence (e.g., NNN) and at least a portion of the universal sample index sequence using labeled multivalent molecules provides a balanced ratio of fluorescent colors that correspond to nucleo-bases adenine, cytosine, guanine, thymine and/or uracil (e.g., see FIG. 26). The labeled multivalent molecules emit fluorescent signals during the sequencing reactions. In some embodiments, the sequencing reaction is conducted on a

sequencing apparatus having a detector that captures fluorescent images from sequencing reactions on the immobilized template molecules. The sequencing apparatus can be configured to relay the fluorescent imaging data captured by the detector to a computer system that is programmed to determine the location (e.g., mapping) of the immobilized template molecules (colonies) on the flowcell. The computer system can generate a map of the locations of the immobilized template molecules based on the fluorescent imaging data of only the random sequence (e.g., NNN), or based on the random sequence (e.g., NNN) and at least a portion of the universal sample index sequence. Thus the few numbers of sequencing cycles used to sequence the random sequence (e.g., NNN) and optionally a portion of the universal sample index sequence can be used to generate a map of the location of the immobilized template molecules. The computer system can be configured to extract the fluorescent color and intensity of only the random sequence (e.g., NNN) or the random sequence (e.g., NNN) and the universal sample index sequence. The computer system can be configured to use the location of a given immobilized template molecule and the fluorescent color and intensity associated with the given template molecule (which were established while sequencing the random sequence) for base calling while sequencing the insert region (110). The computer system can be configured to detect phasing and pre-phasing while sequencing the random sequence (e.g., NNN) and the universal sample index sequence, and the insert region (110). In some embodiments, the balanced ratio of fluorescent colors provided by the random sequence (e.g., NNN) at each sequencing cycle can improve the quality of the data which is processed from the fluorescent images captured by the detector, and can in turn improve the capability by the computer system to determine the location of the immobilized template molecules on the flowcell, and the color and intensity, all of which can improve base calling accuracy and quality scores of the sequenced insert region (110).

#### **A First Embodiment: Order of Sequencing Sample Index Sequences**

**[0433]** In some embodiments, the order of sequencing comprises: **(1)** sequencing the right sample index (170) where the right index comprises a first random sequence (e.g., NNN) and a right universal sample index sequence; **(2)** sequencing the left sample index (160); and **(3)** sequencing the insert region (110). In some embodiments, the left sample index (160) comprises a left universal sample index sequence. In some embodiments, the left sample index (160) comprises a second random sequence (e.g., NNN) and a left universal sample index sequence. In some embodiments, sequencing the right sample index region (170),

including the first random sequence (e.g., NNN) and right universal sample index sequence, may provide enough nucleotide diversity so that sequencing the left sample index (160) can be omitted.

**[0434]** In some embodiments, methods for sequencing the template molecules immobilized to a support, wherein individual template molecules comprise: (i) a universal binding sequence for a first surface primer (120), (ii) a left sample index sequence (160) having a universal left sample index sequence, (iii) a universal binding sequence for a forward sequencing primer (140), (iv) a sequence of interest (110), (v) a universal binding sequence for a reverse sequencing primer (150), (vi) a right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), and (vii) a universal binding sequence for a second surface primer (130) (e.g., see FIGS. 5 and 6), wherein the method comprises **step (a)**: hybridizing the template molecules with a first plurality of soluble sequencing primers that hybridize to the universal binding sequence for a reverse sequencing primer (150) and sequencing the right sample index sequence (170) including sequencing the short random sequence (e.g., NNN) and the right universal sample index sequence thereby generating a first plurality of sample index extension products that are hybridized to the immobilized template molecules, wherein the first plurality of sample index extension products are complementary to the right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170).

**[0435]** In some embodiments, the methods for sequencing further comprise **step (b)**: removing the first plurality of sample index extension products and retaining the immobilized template molecules.

**[0436]** In some embodiments, the methods for sequencing further comprise **step (c)**: hybridizing the retained immobilized template molecules with a second plurality of soluble sequencing primers that hybridize to the universal binding sequence for the first surface primer (120) and sequencing the left sample index sequence (160) thereby generating a second plurality of sample index extension products that are hybridized to the immobilized template molecules, wherein the second plurality of sample index extension products are complementary to the left sample index sequence (160) having a left universal sample index sequence.

**[0437]** In some embodiments, the methods for sequencing further comprise **step (d)**: removing the second plurality of sample index extension products and retaining the immobilized template molecules.

**[0438]** In some embodiments, the methods for sequencing further comprise **step (e)**: hybridizing the retained immobilized template molecules with a third plurality of soluble sequencing primers that hybridize to the universal binding sequence for the forward sequencing primer (140) and sequencing the insert region (110) thereby generating a plurality of insert extension products that are hybridized to the immobilized template molecules, wherein the plurality of insert extension products are complementary to the sequence of interest (110).

**[0439]** In some embodiments, the methods for sequencing further comprise **step (f1)**: assigning the sequence of (i) the insert region (110) to (ii) the right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), thereby identifying the insert region as being obtained from a first source.

**[0440]** In some embodiments, the methods for sequencing further comprise **step (f2)**: assigning the sequence of (i) the insert region (110) to (ii) the right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), and (iii) the left sample index sequence (160), thereby identifying the insert region as being obtained from a first source.

**[0441]** In some embodiments, the removing of the plurality of sequencing extension products of steps (b) and (d) can be conducted using a denaturation reagent comprising SSC (e.g., saline-sodium citrate) buffer with or without formamide, at a temperature that promotes nucleic acid denaturation such as for example 50 – 90 °C.

**[0442]** In some embodiments, the sequencing of steps (a), (c) and (e) include conducting any of the sequencing methods described herein that employ sequencing polymerases and detectably labeled nucleotide analogs. In some embodiments, the sequencing of steps (a), (c) and (e) include conducting any of the two-stage sequencing methods described herein that employ sequencing polymerases, detectably labeled multivalent molecules, and nucleotide analogs. In some embodiments, the sequencing of steps (a), (c) and (e) include conducting any of the sequencing-by-binding methods described herein.

**[0443]** In some embodiments, the density of the plurality of template molecules immobilized to the support is about  $10^2 - 10^{15}$  per  $\text{mm}^2$ . In some embodiments, the plurality of template molecules are immobilized at random locations on the support. In some embodiments, the plurality of template molecules are immobilized on the support in a predetermined pattern.

## **A Second Embodiment: Order of Sequencing Sample Index Sequences**

[0444] In some embodiments, the order of sequencing comprises: **(1)** sequencing the right sample index (170) where the right index comprises a random sequence (e.g., NNN) and a universal sample index sequence; **(2)** sequencing the insert region (110); and **(3)** sequencing the left sample index (160). In some embodiments, the left sample index (160) comprises a left universal sample index sequence. In some embodiments, the left sample index (160) comprises a second random sequence (e.g., NNN) and a left universal sample index sequence. In some embodiments, sequencing the right sample index region (170), including the first random sequence (e.g., NNN) and right universal sample index sequence, may provide enough nucleotide diversity so that sequencing the left sample index (160) can be omitted.

[0445] In some embodiments, methods for sequencing the template molecules immobilized to a support, wherein individual template molecules comprise: (i) a universal binding sequence for a first surface primer (120), (ii) a left sample index sequence (160) having a left universal sample index sequence (iii) a universal binding sequence for a forward sequencing primer (140), (iv) a sequence of interest (110), (v) a universal binding sequence for a reverse sequencing primer (150), (vi) a right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), and (vii) a universal binding sequence for a second surface primer (130) (e.g., see FIGS. 5 and 6), wherein the method comprises **step (a)**: hybridizing the template molecules with a first plurality of soluble sequencing primers that hybridize to the universal binding sequence for a reverse sequencing primer (150) and sequencing the right sample index sequence (170) including sequencing the short random sequence (e.g., NNN) and the right universal sample index sequence thereby generating a first plurality of sample index extension products that are hybridized to the immobilized template molecules, wherein the first plurality of sample index extension products are complementary to the right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170).

[0446] In some embodiments, the methods for sequencing further comprise **step (b)**: removing the first plurality of sample index extension products and retaining the immobilized template molecules.

[0447] In some embodiments, the methods for sequencing further comprise **step (c)**: hybridizing the retained immobilized template molecules with a second plurality of soluble sequencing primers that hybridize to the universal binding sequence for a forward sequencing primer (140) and sequencing the insert region (110) thereby generating a plurality of insert

extension products that are hybridized to the immobilized template molecules, wherein the plurality of insert extension products are complementary to the sequence of interest (110).

**[0448]** In some embodiments, the methods for sequencing further comprise **step (d)**: removing the plurality of insert extension products and retaining the immobilized template molecules.

**[0449]** In some embodiments, the methods for sequencing further comprise **step (e)**: hybridizing the retained immobilized template molecules with a third plurality of soluble sequencing primers that hybridize to the universal binding sequence for a first surface primer (120) and sequencing the left sample index sequence (160) thereby generating a second plurality of sample index extension products that are hybridized to the immobilized template molecules, wherein the second plurality of sample index extension products are complementary to the left sample index sequence (160) having a left universal sample index sequence.

**[0450]** In some embodiments, the methods for sequencing further comprise **step (f1)**: assigning the sequence of (i) the insert region (110) to (ii) the right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), thereby identifying the insert region as being obtained from a first source.

**[0451]** In some embodiments, the methods for sequencing further comprise **step (f2)**: assigning the sequence of (i) the insert region (110) to (ii) the right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), and (iii) the left sample index sequence (160), thereby identifying the insert region as being obtained from a first source.

**[0452]** In some embodiments, the removing of the plurality of sequencing extension products of steps (b) and (d) can be conducted using a denaturation reagent comprising SSC (e.g., saline-sodium citrate) buffer with or without formamide, at a temperature that promotes nucleic acid denaturation such as for example 50 – 90 °C.

**[0453]** In some embodiments, the sequencing of steps (a), (c) and (e) include conducting any of the sequencing methods described herein that employ sequencing polymerases and detectably labeled nucleotide analogs. In some embodiments, the sequencing of steps (a), (c) and (e) include conducting any of the two-stage sequencing methods described herein that employ sequencing polymerases, detectably labeled multivalent molecules, and nucleotide analogs. In some embodiments, the sequencing of steps (a), (c) and (e) include conducting any of the sequencing-by-binding methods described herein.

[0454] In some embodiments, the density of the plurality of template molecules immobilized to the support is about  $10^2 - 10^{15}$  per  $\text{mm}^2$ . In some embodiments, the plurality of template molecules are immobilized at random locations on the support. In some embodiments, the plurality of template molecules are immobilized on the support in a predetermined pattern.

### A Third Embodiment: Order of Sequencing Sample Index Sequences

[0455] In some embodiments, the order of sequencing comprises: **(1)** sequencing the insert region (110); **(2)** sequencing the right sample index (170) where the right index comprises a random sequence (e.g., NNN) and a universal sample index sequence; and **(3)** sequencing the left sample index (160). In some embodiments, the left sample index (160) comprises a left universal sample index sequence. In some embodiments, the left sample index (160) comprises a second random sequence (e.g., NNN) and a left universal sample index sequence. In some embodiments, sequencing the right sample index region (170), including the first random sequence (e.g., NNN) and right universal sample index sequence, may provide enough nucleotide diversity so that sequencing the left sample index (160) can be omitted.

[0456] In some embodiments, methods for sequencing the template molecules immobilized to a support, wherein individual template molecules comprise: (i) a universal binding sequence for a first surface primer (120), (ii) a left sample index sequence (160) having a left universal sample index sequence (iii) a universal binding sequence for a forward sequencing primer (140), (iv) a sequence of interest (110), (v) a universal binding sequence for a reverse sequencing primer (150), (vi) a right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), and (vii) a universal binding sequence for a second surface primer (130) (e.g., see FIGS. 5 and 6), wherein the method comprises **step (a)**: hybridizing the template molecules with a first plurality of soluble sequencing primers that hybridize to the universal binding sequence for a forward sequencing primer (140) and sequencing the insert region (110) thereby generating a plurality of insert extension products that are hybridized to the immobilized template molecules, wherein the plurality of insert extension products are complementary to the sequence of interest (110).

[0457] In some embodiments, the methods for sequencing further comprise **step (b)**: removing the plurality of insert extension products and retaining the immobilized template molecules.

**[0458]** In some embodiments, the methods for sequencing further comprise **step (c)**: hybridizing the template molecules with a second plurality of soluble sequencing primers that hybridize to the universal binding sequence for a reverse sequencing primer (150) and sequencing the right sample index sequence (170) including sequencing the short random sequence (e.g., NNN) and the right universal sample index sequence thereby generating a first plurality of sample index extension products that are hybridized to the immobilized template molecules, wherein the first plurality of sample index extension products are complementary to the right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170).

**[0459]** In some embodiments, the methods for sequencing further comprise **step (d)**: removing the first plurality of sample index extension products and retaining the immobilized template molecules.

**[0460]** In some embodiments, the methods for sequencing further comprise **step (e)**: hybridizing the retained immobilized template molecules with a third plurality of soluble sequencing primers that hybridize to the universal binding sequence for a first surface primer (120) and sequencing the left sample index sequence (160) thereby generating a second plurality of sample index extension products that are hybridized to the immobilized template molecules, wherein the second plurality of sample index extension products are complementary to the left sample index sequence (160) having a left universal sample index sequence.

**[0461]** In some embodiments, the methods for sequencing further comprise **step (f1)**: assigning the sequence of (i) the insert region (110) to (ii) the right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), thereby identifying the insert region as being obtained from a first source.

**[0462]** In some embodiments, the methods for sequencing further comprise **step (f2)**: assigning the sequence of (i) the insert region (110) to (ii) the right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), and (iii) the left sample index sequence (160), thereby identifying the insert region as being obtained from a first source.

**[0463]** In some embodiments, the removing of the plurality of sequencing extension products of steps (b) and (d) can be conducted using a denaturation reagent comprising SSC (e.g., saline-sodium citrate) buffer with or without formamide, at a temperature that promotes nucleic acid denaturation such as for example 50 – 90 °C.

**[0464]** In some embodiments, the sequencing of steps (a), (c) and (e) include conducting any of the sequencing methods described herein that employ sequencing polymerases and detectably labeled nucleotide analogs. In some embodiments, the sequencing of steps (a), (c) and (e) include conducting any of the two-stage sequencing methods described herein that employ sequencing polymerases, detectably labeled multivalent molecules, and nucleotide analogs. In some embodiments, the sequencing of steps (a), (c) and (e) include conducting any of the sequencing-by-binding methods described herein.

**[0465]** In some embodiments, the density of the plurality of template molecules immobilized to the support is about  $10^2 - 10^{15}$  per  $\text{mm}^2$ . In some embodiments, the plurality of template molecules are immobilized at random locations on the support. In some embodiments, the plurality of template molecules are immobilized on the support in a predetermined pattern.

#### **A Fourth Embodiment: Order of Sequencing**

**[0466]** In some embodiments, the order of sequencing comprises: **(1)** sequencing the first 3-5 bases of the insert region (110); **(2)** sequencing the right sample index (170) where the right index comprises an optional random sequence (e.g., NNN) and a universal sample index sequence; and **(3)** sequencing the left sample index (160). In some embodiments, the left sample index (160) comprises a left universal sample index sequence. In some embodiments, the left sample index (160) comprises a second random sequence (e.g., NNN) and a left universal sample index sequence. In some embodiments, sequencing the first 3-5 bases of the insert region (110) may provide enough sequence diversity so that the right sample index (170) and the left sample index (160) do not include a short random sequence (e.g., NNN). In some embodiments, sequencing the right sample index region (170), including the first random sequence (e.g., NNN) and right universal sample index sequence, may provide enough nucleotide diversity so that sequencing the left sample index (160) can be omitted.

**[0467]** In some embodiments, methods for sequencing the template molecules immobilized to a support, wherein individual template molecules comprise: (i) a universal binding sequence for a first surface primer (120), (ii) a left sample index sequence (160) having a left universal sample index sequence (iii) a universal binding sequence for a forward sequencing primer (140), (iv) a sequence of interest (110), (v) a universal binding sequence for a reverse sequencing primer (150), (vi) a right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), and (vii) a universal binding sequence for a second surface primer (130) (e.g., see FIGS. 5 and 6),

wherein the method comprises **step (a)**: hybridizing the template molecules with a first plurality of soluble sequencing primers that hybridize to the universal binding sequence for a forward sequencing primer (140) and sequencing the first 3-5 bases of the insert region (110) thereby generating a plurality of insert extension products that are hybridized to the immobilized template molecules, wherein the plurality of insert extension products are complementary to the sequence of interest (110). The sequence of the first 3-5 bases of the insert region (110) may provide sufficient sequence diversity and color balance for polony mapping and template registration.

**[0468]** In some embodiments, the methods for sequencing further comprise **step (b)**: removing the plurality of insert extension products and retaining the immobilized template molecules.

**[0469]** In some embodiments, the methods for sequencing further comprise **step (c)**: hybridizing the template molecules with a second plurality of soluble sequencing primers that hybridize to the universal binding sequence for a reverse sequencing primer (150) and sequencing the right sample index sequence (170) including sequencing the short random sequence (e.g., NNN) if present and the right universal sample index sequence thereby generating a first plurality of sample index extension products that are hybridized to the immobilized template molecules, wherein the first plurality of sample index extension products are complementary to the right sample index sequence (170).

**[0470]** In some embodiments, the methods for sequencing further comprise **step (d)**: removing the first plurality of sample index extension products and retaining the immobilized template molecules.

**[0471]** In some embodiments, the methods for sequencing further comprise **step (e)**: hybridizing the retained immobilized template molecules with a third plurality of soluble sequencing primers that hybridize to the universal binding sequence for a first surface primer (120) and sequencing the left sample index sequence (160) thereby generating a second plurality of sample index extension products that are hybridized to the immobilized template molecules, wherein the second plurality of sample index extension products are complementary to the left sample index sequence (160) having a left universal sample index sequence.

**[0472]** In some embodiments, the methods for sequencing further comprise **step (f)**: removing the second plurality of sample index extension products and retaining the immobilized template molecules.

**[0473]** In some embodiments, the methods for sequencing further comprise **step (g)**: hybridizing the template molecules with a fourth plurality of soluble sequencing primers that hybridize to the universal binding sequence for a forward sequencing primer (140) and sequencing the full length of the insert region (110) thereby generating a plurality of full length insert extension products that are hybridized to the immobilized template molecules, wherein the plurality of full length insert extension products are complementary to the sequence of interest (110).

**[0474]** In some embodiments, the methods for sequencing further comprise **step (h1)**: assigning the full length sequence of (i) the insert region (110) to (ii) the right sample index sequence (170), thereby identifying the insert region as being obtained from a first source.

**[0475]** In some embodiments, the methods for sequencing further comprise **step (h2)**: assigning the full length sequence of (i) the insert region (110) to (ii) the right sample index sequence (170), and (iii) the left sample index sequence (160), thereby identifying the insert region as being obtained from a first source.

**[0476]** In some embodiments, the removing of the plurality of sequencing extension products of steps (b), (d) and (f) can be conducted using a denaturation reagent comprising SSC (e.g., saline-sodium citrate) buffer with or without formamide, at a temperature that promotes nucleic acid denaturation such as for example 50 – 90 °C.

**[0477]** In some embodiments, the sequencing of steps (a), (c), (e) and (g) include conducting any of the sequencing methods described herein that employ sequencing polymerases and detectably labeled nucleotide analogs. In some embodiments, the sequencing of steps (a), (c), (e) and (g) include conducting any of the two-stage sequencing methods described herein that employ sequencing polymerases, detectably labeled multivalent molecules, and nucleotide analogs. In some embodiments, the sequencing of steps (a), (c), (e) and (g) include conducting any of the sequencing-by-binding methods described herein.

**[0478]** In some embodiments, the density of the plurality of template molecules immobilized to the support is about  $10^2 - 10^{15}$  per  $\text{mm}^2$ . In some embodiments, the plurality of template molecules are immobilized at random locations on the support. In some embodiments, the plurality of template molecules are immobilized on the support in a predetermined pattern.

#### **A Fifth Embodiment: Order of Sequencing**

[0479] In some embodiments, the order of sequencing comprises: **(1)** sequencing the first 3-5 bases of the insert region (110) of the immobilized template molecule (e.g., sequencing in a forward direction); **(2)** sequencing the right sample index (170) where the right index comprises a first random sequence (e.g., NNN) and a right universal sample index sequence; **(3)** sequencing the left sample index (160); **(4)** conducting a pairwise turn reaction so that the immobilized template molecule is replaced with an immobilized strand that is complementary to the template molecule; and **(5)** sequencing the full-length of the insert region (110) of the immobilized complementary strand (e.g., sequencing in the reverse direction). In some embodiments, the sequences of the first 3-5 bases of the insert region (110) of a population of library molecules may provide enough sequence diversity for improved base-calling accuracy. In some embodiments, the left sample index (160) comprises a left universal sample index sequence. In some embodiments, the left sample index (160) comprises a second random sequence (e.g., NNN) and a left universal sample index sequence. In some embodiments, sequencing the right sample index region (170), including the first random sequence (e.g., NNN) and right universal sample index sequence, may provide enough nucleotide diversity so that sequencing the left sample index (160) can be omitted.

[0480] In some embodiments, methods for sequencing the template molecules immobilized to a support, wherein individual template molecules are covalently linked to an immobilized capture primer that lacks uracil bases, and individual template molecules comprise randomly-distributed uracil bases, and individual template molecules comprise: (i) a universal binding sequence for a first surface primer (120), (ii) a left sample index sequence (160) having a left universal sample index sequence (iii) a universal binding sequence for a forward sequencing primer (140), (iv) a sequence of interest (110), (v) a universal binding sequence for a reverse sequencing primer (150), (vi) a right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), and (vii) a universal binding sequence for a second surface primer (130) (e.g., see FIGS. 5 and 6), wherein the method comprises **step (a)**: hybridizing the template molecules with a first plurality of soluble sequencing primers (e.g., forward sequencing primers) that hybridize to the universal binding sequence for a forward sequencing primer (140) and sequencing the first 3-5 bases of the insert region (110) thereby generating a plurality of insert extension products that are hybridized to the immobilized template molecules, wherein the plurality of insert extension products are complementary to the sequence of interest (110).

[0481] In some embodiments, the methods for sequencing further comprise **step (b)**: removing the plurality of insert extension products and retaining the immobilized template molecules.

[0482] In some embodiments, the methods for sequencing further comprise **step (c)**: hybridizing the template molecules with a second plurality of soluble sequencing primers that hybridize to the universal binding sequence for a reverse sequencing primer (150) and sequencing the right sample index sequence (170) including sequencing the short random sequence (e.g., NNN) and the right universal sample index sequence thereby generating a first plurality of sample index extension products that are hybridized to the immobilized template molecules, wherein the first plurality of sample index extension products are complementary to the right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170).

[0483] In some embodiments, the methods for sequencing further comprise **step (d)**: removing the first plurality of sample index extension products and retaining the immobilized template molecules.

[0484] In some embodiments, the methods for sequencing further comprise **step (e)**: hybridizing the retained immobilized template molecules with a third plurality of soluble sequencing primers that hybridize to the universal binding sequence for a first surface primer (120) and sequencing the left sample index sequence (160) thereby generating a second plurality of sample index extension products that are hybridized to the immobilized template molecules, wherein the second plurality of sample index extension products are complementary to the left sample index sequence (160) having a left universal sample index sequence.

[0485] In some embodiments, the methods for sequencing further comprise **step (f)**: replacing the second plurality of sample index extension products that are hybridized to the immobilized template molecules by conducting a primer extension reaction using strand-displacing polymerases and a plurality of nucleotides to generate an extension product that is hybridized to the immobilized template molecules including the immobilized capture primer.

[0486] In some embodiments, the methods for sequencing further comprise **step (g)**: removing the immobilized template molecules by generating abasic sites in the immobilized template molecules at the uracil sites and generating gaps at the abasic sites thereby generating gap-containing template molecules while retaining the extension products that were generated in step (f) where individual extension products are retained by being

hybridized to an immobilized capture primer. In some embodiments, pairwise turn is achieved by conducting steps (g) and (h).

**[0487]** In some embodiments, the methods for sequencing further comprise **step (h)**: hybridizing the retained extension products with a fourth plurality of soluble sequencing primers (e.g., reverse sequencing primers) that hybridize to universal binding sequence for a reverse sequencing primer (150) and sequencing the insert region (110) (e.g., sequencing at least a portion or the full length of the insert region (110)).

**[0488]** In some embodiments, the methods for sequencing further comprise **step (i1)**: assigning the sequence of (i) the insert region (110) to (ii) the right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), thereby identifying the insert region as being obtained from a first source.

**[0489]** In some embodiments, the methods for sequencing further comprise **step (i2)**: assigning the sequence of (i) the insert region (110) to (ii) the right sample index sequence having a short random sequence (e.g., NNN) linked directly to a right universal sample index sequence (170), and (iii) the left sample index sequence (160), thereby identifying the insert region as being obtained from a first source.

**[0490]** In some embodiments, the removing of the plurality of sequencing extension products of steps (b) and (d) can be conducted using a denaturation reagent comprising SSC (e.g., saline-sodium citrate) buffer with or without formamide, at a temperature that promotes nucleic acid denaturation such as for example 50 – 90 °C.

**[0491]** In some embodiments, the sequencing of steps (a), (c), (e) and (h) include conducting any of the sequencing methods described herein that employ sequencing polymerases and detectably labeled nucleotide analogs. In some embodiments, the sequencing of steps (a), (c), (e) and (h) include conducting any of the two-stage sequencing methods described herein that employ sequencing polymerases, detectably labeled multivalent molecules, and nucleotide analogs. In some embodiments, the sequencing of steps (a), (c), (e) and (h) include conducting any of the sequencing-by-binding methods described herein.

**[0492]** In some embodiments, the density of the plurality of template molecules immobilized to the support is about  $10^2 - 10^{15}$  per  $\text{mm}^2$ . In some embodiments, the plurality of template molecules are immobilized at random locations on the support. In some embodiments, the plurality of template molecules are immobilized on the support in a predetermined pattern.

**Sequencing 3-mer Random Sequences to Generate a Polony Map**

**[0493]** The present disclosure provides methods for sequencing nucleic acids comprising: **(a)** providing a plurality of nucleic acid template molecules immobilized on a support (e.g., immobilized at random or pre-determined locations), wherein individual immobilized template molecules comprise an insert sequence region and one sample index, wherein each sample index comprises a 3-mer random sequence joined to a universal sample index sequence which identifies the sample source of the insert sequence, wherein different immobilized template molecules have a different 3-mer random sequence and the same universal sample index sequence, and wherein the immobilized template molecules have different insert sequences; **(b)** conducting three cycles of polymerase-mediated sequencing reactions of the 3-mer random sequence of the plurality of immobilized template molecules using a plurality of detectably labeled nucleotide reagents comprising a mixture of different types of nucleobases A, G, C and T/U, wherein the nucleotide reagents comprise a different detectable color label that corresponds with each different type of nucleobase, wherein the three cycles of sequencing include detecting and imaging the optical color signals emitted from the detectably labeled nucleotide reagents that are bound to the immobilized template molecules thereby determining the sequences of the 3-mer random sequences in individual template molecules of the plurality of immobilized template molecules, and wherein a balanced diversity of nucleobases of A, G, C and T/U is detected and imaged in each of the first, second and third sequencing cycles among the plurality of immobilized template molecules; and **(c)** generating a map of the locations of the plurality of immobilized template molecules using the images obtained in step (b), wherein the sequence of the insert region is not used to generate the map.

**[0494]** In some embodiments, in the methods for sequencing nucleic acids, the balanced diversity of step (b) is about 5-85%, or about 5-60%, or about 10-50%, or about 15-55%, or about 25-75% of each of the nucleobases A, G, C and T/U that are detected and imaged in each of the first, second and third sequencing cycles.

**[0495]** In some embodiments, in the methods for sequencing nucleic acids, the method further comprises: **(a)** sequencing the universal sample index sequence of the plurality of immobilized template molecules; **(b)** sequencing the insert sequence region of the plurality of immobilized template molecules; and **(c)** assigning the insert sequence of a given template molecule obtained in step (b) with the universal sample index sequence from the same given template molecule obtained in step (a), thereby identifying the sample source of the given insert sequence.

**[0496]** In some embodiments, in the methods for sequencing nucleic acids, the plurality of nucleic acid template molecules further comprise a second sample index which comprises a second universal sample index sequence which identifies the sample source of the insert sequence and the second sample index lacks a random sequence.

**[0497]** In some embodiments, in the methods for sequencing nucleic acids, the method further comprises: **(a)** sequencing the 3-mer random sequence of the plurality of immobilized template molecules to obtain a balanced diversity of nucleo-bases of A, G, C and T/U that are detected and imaged in each of the first, second and third sequencing cycles to generate a map of the locations of the plurality of immobilized template molecules; **(b)** sequencing the first universal sample index sequence of the plurality of immobilized template molecules; **(c)** sequencing the second universal sample index sequence of the plurality of immobilized template molecules; **(d)** sequencing the insert sequence region of the plurality of immobilized template molecules; and **(e)** assigning the insert sequence of a given template molecule obtained in step (c) with the first and second universal sample index sequences from the same given template molecule obtained in steps (a) and (b), thereby identifying the sample source of the given insert sequence.

**[0498]** The present disclosure provides methods for sequencing nucleic acids comprising: **(a)** providing a plurality of nucleic acid template molecules immobilized on a support (e.g., immobilized at random or pre-determined locations), wherein individual immobilized template molecules comprise an insert sequence region and one sample index, wherein each sample index comprises a 3-mer random sequence joined to a universal sample index sequence which identifies the sample source of the insert sequence, wherein the universal sample index sequence comprises 3-20 nucleotides, wherein different immobilized template molecules have a different 3-mer random sequence and the same universal sample index sequence, and wherein the immobilized template molecules have different insert sequences; **(b)** conducting four cycles of polymerase-mediated sequencing reactions of the 3-mer random sequence and the first base position of the universal sample index sequence of the plurality of immobilized template molecules using a plurality of detectably labeled nucleotide reagents comprising a mixture of different types of nucleo-bases A, G, C and T/U, wherein the nucleotide reagents comprise a different detectable color label that corresponds with each different type of nucleo-base, wherein the four cycles of sequencing include detecting and imaging the optical color signals emitted from the detectably labeled nucleotide reagents that are bound to the immobilized template molecules thereby determining the sequences of the 3-mer random sequences and the first base position of the universal sample index sequences in

individual template molecules of the plurality of immobilized template molecules, and wherein a balanced diversity of nucleobases of A, G, C and T/U is detected and imaged in each of the first, second, third and fourth sequencing cycles among the plurality of immobilized template molecules; and **(c)** generating a map of the locations of the plurality of immobilized template molecules using the images of the four cycles of polymerase-mediated sequencing reactions obtained in step (b), wherein the sequence of the insert region is not used to generate the map.

**[0499]** In some embodiments, in the methods for sequencing nucleic acids, the balanced diversity of step (b) is about 5-85%, or about 5-60%, or about 10-50%, or about 15-55%, or about 25-75% of each of the nucleobases A, G, C and T/U that are detected and imaged in each of the first, second, third and fourth sequencing cycles.

**[0500]** In some embodiments, in the methods for sequencing nucleic acids, the method further comprises: **(a)** sequencing the remaining base positions of the universal sample index sequence of the plurality of immobilized template molecules; **(b)** sequencing the insert sequence region of the plurality of immobilized template molecules; and **(c)** assigning the insert sequence of a given template molecule obtained in step (b) with the universal sample index sequence from the same given template molecule obtained in step (a), thereby identifying the sample source of the given insert sequence.

**[0501]** In some embodiments, in the methods for sequencing nucleic acids, the plurality of nucleic acid template molecules further comprise: a second sample index which comprises a second universal sample index sequence which identifies the sample source of the insert sequence and the second sample index lacks a random sequence.

**[0502]** In some embodiments, in the methods for sequencing nucleic acids, the method further comprises: **(a)** sequencing the 3-mer random sequence and the first base position of the universal sample index sequence of the plurality of immobilized template molecules to obtain a balanced diversity of nucleobases of A, G, C and T/U that are detected and imaged in each of the first, second, third and fourth sequencing cycles to generate a map of the locations of the plurality of immobilized template molecules; **(b)** sequencing the remaining base positions of the first universal sample index sequence of the plurality of immobilized template molecules; **(c)** sequencing the second universal sample index sequence of the plurality of immobilized template molecules; **(d)** sequencing the insert sequence region of the plurality of immobilized template molecules; and **(e)** assigning the insert sequence of a given template molecule obtained in step (c) with the first and second universal sample index

sequences from the same given template molecule obtained in steps (a) and (b), thereby identifying the sample source of the given insert sequence.

**[0503]** In some embodiments, in any of the methods for sequencing nucleic acids, the support comprises a glass or plastic substrate. In some embodiments, the support is configured on a flowcell channel, a flow cell, or a capillary lumen. In some embodiments, the support is passivated with at least one hydrophilic polymer coating having a water contact angle of not more than 45 degrees. In some embodiments, the at least one hydrophilic polymer coating comprises a molecule selected from a group consisting of polyethylene glycol (PEG), poly(vinyl alcohol) (PVA), poly(vinyl pyridine), poly(vinyl pyrrolidone) (PVP), poly(acrylic acid) (PAA), polyacrylamide, poly(N-isopropylacrylamide) (PNIPAM), poly(methyl methacrylate) (PMA), poly(2-hydroxyethyl methacrylate) (HEMA), poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), polyglutamic acid (PGA), poly-lysine, poly-glucoside, streptavidin, and dextran. In some embodiments, the at least one hydrophilic polymer coating comprises branched hydrophilic polymer molecules having at least four branches. In some embodiments, the at least one hydrophilic polymer coating comprises polymer molecules having a molecular weight of at least 1000 Daltons.

**[0504]** In some embodiments, in any of the methods for sequencing nucleic acids, the immobilized template molecules comprise a plurality of immobilized concatemer molecules having tandem repeat sequences of the insert sequence and the one sample index. In some embodiments, the immobilized template molecules comprise a plurality of different clustered template molecules having one copy of the insert sequence and one copy of the one sample index, wherein the clustered template molecules are generated via bridge amplification. In some embodiments, the density of the immobilized nucleic acid template molecules positioned at random or pre-determined locations on the support is  $10^4 - 10^8$  per  $\text{mm}^2$ . In some embodiments, the sample source of the insert sequences is genomic DNA, double-stranded cDNA or cell free circulating DNA.

**[0505]** In some embodiments, in any of the methods for sequencing nucleic acids, the detectably labeled nucleotide reagents comprise nucleotides each comprising an aromatic nucleo-base, a five carbon sugar moiety, 1-10 phosphate groups, and a fluorophore. In some embodiments, the detectably labeled nucleotide reagents comprise nucleotides each comprising an aromatic nucleo-base, a five carbon sugar moiety having a chain terminating group at the 3' carbon sugar position, 1-10 phosphate groups, and a fluorophore. In some embodiments, the detectably labeled nucleotide reagents comprise multivalent molecules each comprising (1) a core, (2) a plurality of nucleotide arms, and (3) at least one

fluorophore, wherein individual nucleotide arms 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, wherein the spacer is attached to the linker, wherein the linker is attached to the nucleotide unit.

**[0506]** In some embodiments, in any of the methods for sequencing nucleic acids, the detectably labeled nucleotide reagents that are bound to the immobilized template molecules in step (b) comprise individual immobilized template molecules hybridized to a sequencing primer to form a duplex, and the duplex is bound to a polymerase to form a complexed polymerase, and the complexed polymerase is bound to a detectably labeled nucleotide reagent. In some embodiments, the complexed polymerase is bound to a detectably labeled nucleotide reagent under a condition suitable for binding the detectably labeled nucleotide reagent to the complexed polymerase and incorporating the detectably labeled nucleotide into the hybridized sequencing primer, wherein the detectably labeled nucleotide reagent comprises an aromatic nucleobase, a five carbon sugar moiety, 1-10 phosphate groups, and a fluorophore. In some embodiments, the complexed polymerase is bound to a detectably labeled nucleotide reagent under a condition suitable for binding the detectably labeled nucleotide reagent to the complexed polymerase and incorporating the detectably labeled nucleotide into the hybridized sequencing primer, wherein the detectably labeled nucleotide reagent comprises an aromatic nucleobase, a five carbon sugar moiety having a chain terminating group at the 3' carbon sugar position, 1-10 phosphate groups, and a fluorophore. In some embodiments, the complexed polymerase is bound to a detectably labeled nucleotide reagent under a condition suitable for binding the detectably labeled nucleotide reagent to the complexed polymerase and the condition is suitable for inhibiting nucleotide incorporation, wherein the detectably labeled nucleotide reagent comprises a multivalent molecule which includes **(1)** a core, **(2)** a plurality of nucleotide arms, and **(3)** at least one fluorophore, wherein individual nucleotide arms 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, wherein the spacer is attached to the linker, wherein the linker is attached to the nucleotide unit.

**[0507]** In some embodiments, in any of the methods for sequencing nucleic acids, the immobilized template molecule comprises an immobilized concatemer molecule which is hybridized to a plurality of sequencing primers to form at least a first and second duplex on the same concatemer molecule, wherein the first duplex is bound to a first polymerase and the second duplex is bound to a second polymerase to form first and second complexed

polymerases, and wherein the method comprises: **(a)** contacting a plurality of multivalent molecules to the first and second complexed polymerases on the same concatemer template molecule, wherein individual multivalent molecules include (1) a core, (2) a plurality of nucleotide arms, and (3) at least one fluorophore, wherein individual nucleotide arms 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, and the spacer is attached to the linker, and the linker is attached to the nucleotide unit, wherein the contacting is conducted under conditions suitable to bind a single multivalent molecule from the plurality to the first and second complexed polymerases, wherein a first nucleotide unit of the single multivalent molecule is bound to the first complexed polymerase which includes the first sequencing primer hybridized to a first portion of the concatemer template molecule thereby forming a first binding complex, and wherein a second nucleotide unit of the single multivalent molecule is bound to the second complexed polymerase which includes the second sequencing primer hybridized to a second portion of the concatemer template molecule thereby forming a second binding complex, wherein the first and second binding complexes which are bound to the same multivalent molecule forms an avidity complex, and 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; **(b)** detecting the first and second binding complexes on the same concatemer template molecule; **(c)** imaging the optical color signals emitted from the detectably labeled multivalent molecule which forms the first and second binding complexes on the same concatemer template molecule; and **(d)** identifying the first nucleotide unit in the first binding complex thereby determining the sequence of the first portion of the concatemer template molecule, and identifying the second nucleotide unit in the second binding complex thereby determining the sequence of the second portion of the concatemer template molecule.

### **Multiplex Sequencing 3-mer Random Sequences to Generate a Polony Map**

**[0508]** The present disclosure provides methods for multiplex sequencing nucleic acids comprising: **(a)** providing a first plurality of library molecules each molecule in the plurality comprising (i) an insert sequence region derived from a first sample source, (ii) a first sample index having a 3-mer random sequence joined to a first universal sample index sequence, and (iii) a second sample index having a second universal sample index sequence which lacks a random sequence, wherein the combination of the first and second universal sample index sequences uniquely identifies a first sample source of the insert sequence, wherein different

first library molecules have a different 3-mer random sequence and have a different insert sequence; **(b)** providing a second plurality of library molecules each molecule in the plurality comprising (i) an insert sequence region derived from a second sample source, (ii) a third sample index having a 3-mer random sequence joined to a third universal sample index sequence, and (iii) a fourth sample index having a fourth universal sample index sequence which lacks a random sequence, wherein the combination of the third and fourth universal sample index sequences uniquely identifies a second sample source of the insert sequence, wherein different second library molecules have a different 3-mer random sequence and have a different insert sequence; **(c)** pooling the first and second plurality of library molecules; **(d)** distributing the pooled library molecules onto a support and conducting an amplification reaction to generate a plurality of clonally amplified template molecules immobilized to the support (e.g., immobilized at random or pre-determined locations); **(e)** conducting three cycles of polymerase-mediated sequencing reactions of the 3-mer random sequence of the first and third sample indexes using a plurality of detectably labeled nucleotide reagents comprising a mixture of different types of nucleobases A, G, C and T/U, wherein the nucleotide reagents comprise a different detectable color label that corresponds with each different type of nucleobase, wherein the three cycles of sequencing include detecting and imaging the optical color signals emitted from the detectably labeled nucleotide reagents that are bound to the immobilized amplified template molecules thereby determining the sequences of the 3-mer random sequences in individual template molecules of the plurality of immobilized template molecules, and wherein a balanced diversity of nucleobases of A, G, C and T/U is detected and imaged in each of the first, second and third sequencing cycles among the plurality of immobilized amplified template molecules; and **(f)** generating a map of the locations of the plurality of immobilized template molecules using the images obtained in step (e), wherein the sequence of the insert regions are not used to generate the map.

**[0509]** In some embodiments, in the methods for multiplex sequencing nucleic acids, the balanced diversity of step (e) is about 5-85%, or about 5-60%, or about 10-50%, or about 15-55%, or about 25-75% of each of the nucleobases A, G, C and T/U that are detected and imaged in each of the first, second and third sequencing cycles.

**[0510]** In some embodiments, in the methods for multiplex sequencing nucleic acids, the method further comprises: **(a)** sequencing the first universal sample index sequences of the plurality of immobilized template molecules; **(b)** sequencing the second universal sample index sequences of the plurality of immobilized template molecules; **(c)** sequencing the insert sequence regions of the plurality of immobilized template molecules derived from the first

library molecules; and **(d)** assigning the insert sequence of a given template molecule obtained in step (c) with the first and second universal sample index sequences from the same given template molecule thereby identifying the first sample source of the given insert sequence.

**[0511]** In some embodiments, in the methods for multiplex sequencing nucleic acids, the method further comprises: **(a)** sequencing the third universal sample index sequences of the plurality of immobilized template molecules; **(b)** sequencing the fourth universal sample index sequences of the plurality of immobilized template molecules; **(c)** sequencing the insert sequence regions of the plurality of immobilized template molecules derived from the second library molecules; and **(d)** assigning the insert sequence of a given template molecule obtained in step (c) with the third and fourth universal sample index sequences from the same given template molecule thereby identifying the second sample source of the given insert sequence.

**[0512]** The present disclosure provides methods for multiplex sequencing nucleic acids comprising: **(a)** providing a first plurality of library molecules each molecule in the plurality comprising (i) an insert sequence region derived from a first sample source, (ii) a first sample index having a 3-mer random sequence joined to a first universal sample index sequence, and (iii) a second sample index having a second universal sample index sequence which lacks a random sequence, wherein the combination of the first and second universal sample index sequences uniquely identifies a first sample source of the insert sequence, wherein the first universal sample index sequence comprises 3-20 nucleotides, wherein different first library molecules have a different 3-mer random sequence and have a different insert sequence; **(b)** providing a second plurality of library molecules each molecule in the plurality comprising (i) an insert sequence region derived from a second sample source, (ii) a third sample index having a 3-mer random sequence joined to a third universal sample index sequence, and (iii) a fourth sample index having a fourth universal sample index sequence which lacks a random sequence, wherein the combination of the third and fourth universal sample index sequences uniquely identifies a second sample source of the insert sequence, wherein the third universal sample index sequence comprises 3-20 nucleotides, wherein different second library molecules have a different 3-mer random sequence and have a different insert sequence; **(c)** pooling the first and second plurality of library molecules; **(d)** distributing the pooled library molecules onto a support and conducting an amplification reaction to generate a plurality of clonally amplified template molecules immobilized to the support (e.g., immobilized at random or pre-determined locations); **(e)** conducting four cycles of polymerase-mediated

sequencing reactions of the 3-mer random sequence of the first and third sample indexes and sequencing the first base position of the first and third universal sample index sequences using a plurality of detectably labeled nucleotide reagents comprising a mixture of different types of nucleobases A, G, C and T/U, wherein the nucleotide reagents comprise a different detectable color label that corresponds with each different type of nucleobase, wherein the three cycles of sequencing include detecting and imaging the optical color signals emitted from the detectably labeled nucleotide reagents that are bound to the immobilized amplified template molecules thereby determining the sequences of the 3-mer random sequences in individual template molecules of the plurality of immobilized template molecules, and wherein a balanced diversity of nucleobases of A, G, C and T/U is detected and imaged in each of the first, second, third and fourth sequencing cycles among the plurality of immobilized amplified template molecules; and **(f)** generating a map of the locations of the plurality of immobilized template molecules using the images obtained in step (e), wherein the sequence of the insert regions are not used to generate the map.

**[0513]** In some embodiments, in the methods for multiplex sequencing nucleic acids, the balanced diversity of step (e) is about 5-85%, or about 5-60%, or about 10-50%, or about 15-55%, or about 25-75% of each of the nucleobases A, G, C and T/U that are detected and imaged in each of the first, second, third and fourth sequencing cycles.

**[0514]** In some embodiments, in the methods for multiplex sequencing nucleic acids, the method further comprises: **(a)** sequencing the remaining base positions of the first universal sample index sequences of the plurality of immobilized template molecules; **(b)** sequencing the second universal sample index sequences of the plurality of immobilized template molecules; **(c)** sequencing the insert sequence regions of the plurality of immobilized template molecules derived from the first library molecules; and **(d)** assigning the insert sequence of a given template molecule obtained in step (c) with the first and second universal sample index sequences from the same given template molecule thereby identifying the first sample source of the given insert sequence.

**[0515]** In some embodiments, in the methods for multiplex sequencing nucleic acids, the method further comprises: **(a)** sequencing the remaining base positions of the third universal sample index sequences of the plurality of immobilized template molecules; **(b)** sequencing the fourth universal sample index sequences of the plurality of immobilized template molecules; **(c)** sequencing the insert sequence regions of the plurality of immobilized template molecules derived from the second library molecules; and **(d)** assigning the insert sequence of a given template molecule obtained in step (c) with the third and fourth universal

sample index sequences from the same given template molecule thereby identifying the second sample source of the given insert sequence.

**[0516]** In some embodiments, in any of the methods for multiplex sequencing nucleic acids, the support comprises a glass or plastic substrate. In some embodiments, the support is configured on a flowcell channel, a flow cell, or a capillary lumen. In some embodiments, the support is passivated with at least one hydrophilic polymer coating having a water contact angle of not more than 45 degrees. In some embodiments, the at least one hydrophilic polymer coating comprises a molecule selected from a group consisting of polyethylene glycol (PEG), poly(vinyl alcohol) (PVA), poly(vinyl pyridine), poly(vinyl pyrrolidone) (PVP), poly(acrylic acid) (PAA), polyacrylamide, poly(N-isopropylacrylamide) (PNIPAM), poly(methyl methacrylate) (PMA), poly(2-hydroxyethyl methacrylate) (HEMA), poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), polyglutamic acid (PGA), poly-lysine, poly-glucoside, streptavidin, and dextran. In some embodiments, the at least one hydrophilic polymer coating comprises branched hydrophilic polymer molecules having at least four branches. In some embodiments, the at least one hydrophilic polymer coating comprises polymer molecules having a molecular weight of at least 1000 Daltons.

**[0517]** In some embodiments, in any of the methods for multiplex sequencing nucleic acids, the immobilized template molecules comprise a plurality of immobilized concatemer molecules having tandem repeat sequences of the insert sequence and the one sample index. In some embodiments, the immobilized template molecules comprise a plurality of different clustered template molecules having one copy of the insert sequence and one copy of the one sample index, wherein the clustered template molecules are generated via bridge amplification. In some embodiments, the density of the immobilized nucleic acid template molecules (e.g., immobilized at random or pre-determined locations) on the support is  $10^4 - 10^8$  per  $\text{mm}^2$ . In some embodiments, the sample source of the insert sequences is genomic DNA, double-stranded cDNA or cell free circulating DNA.

**[0518]** In some embodiments, in any of the methods for multiplex sequencing nucleic acids, the detectably labeled nucleotide reagents comprise nucleotides each comprising an aromatic nucleo-base, a five carbon sugar moiety, 1-10 phosphate groups, and a fluorophore. In some embodiments, the detectably labeled nucleotide reagents comprise nucleotides each comprising an aromatic nucleo-base, a five carbon sugar moiety having a chain terminating group at the 3' carbon sugar position, 1-10 phosphate groups, and a fluorophore. In some embodiments, the detectably labeled nucleotide reagents comprise multivalent molecules each comprising **(1)** a core, **(2)** a plurality of nucleotide arms, and **(3)** at least one

fluorophore, wherein individual nucleotide arms 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, wherein the spacer is attached to the linker, wherein the linker is attached to the nucleotide unit.

**[0519]** In some embodiments, in any of the methods for multiplex sequencing nucleic acids, the detectably labeled nucleotide reagents that are bound to the immobilized template molecules in step (e) comprise individual immobilized template molecules hybridized to a sequencing primer to form a duplex, and the duplex is bound to a polymerase to form a complexed polymerase, and the complexed polymerase is bound to a detectably labeled nucleotide reagent. In some embodiments, the complexed polymerase is bound to a detectably labeled nucleotide reagent under a condition suitable for binding the detectably labeled nucleotide reagent to the complexed polymerase and incorporating the detectably labeled nucleotide into the hybridized sequencing primer, wherein the detectably labeled nucleotide reagent comprises an aromatic nucleobase, a five carbon sugar moiety, 1-10 phosphate groups, and a fluorophore. In some embodiments, the complexed polymerase is bound to a detectably labeled nucleotide reagent under a condition suitable for binding the detectably labeled nucleotide reagent to the complexed polymerase and incorporating the detectably labeled nucleotide into the hybridized sequencing primer, wherein the detectably labeled nucleotide reagent comprises an aromatic nucleobase, a five carbon sugar moiety having a chain terminating group at the 3' carbon sugar position, 1-10 phosphate groups, and a fluorophore. In some embodiments, the complexed polymerase is bound to a detectably labeled nucleotide reagent under a condition suitable for binding the detectably labeled nucleotide reagent to the complexed polymerase and the condition is suitable for inhibiting nucleotide incorporation, wherein the detectably labeled nucleotide reagent comprises a multivalent molecule which includes (1) a core, (2) a plurality of nucleotide arms, and (3) at least one fluorophore, wherein individual nucleotide arms 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, wherein the spacer is attached to the linker, wherein the linker is attached to the nucleotide unit.

**[0520]** In some embodiments, in any of the methods for multiplex sequencing nucleic acids, the immobilized template molecule comprises an immobilized concatemer molecule which is hybridized to a plurality of sequencing primers to form at least a first and second duplex on the same concatemer molecule, wherein the first duplex is bound to a first polymerase and the second duplex is bound to a second polymerase to form first and second complexed

polymerases, and wherein the method comprises: **(a)** contacting a plurality of multivalent molecules to the first and second complexed polymerases on the same concatemer template molecule, wherein individual multivalent molecules include (1) a core, (2) a plurality of nucleotide arms, and (3) at least one fluorophore, wherein individual nucleotide arms 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, and the spacer is attached to the linker, and the linker is attached to the nucleotide unit, wherein the contacting is conducted under conditions suitable to bind a single multivalent molecule from the plurality to the first and second complexed polymerases, wherein a first nucleotide unit of the single multivalent molecule is bound to the first complexed polymerase which includes the first sequencing primer hybridized to a first portion of the concatemer template molecule thereby forming a first binding complex, and wherein a second nucleotide unit of the single multivalent molecule is bound to the second complexed polymerase which includes the second sequencing primer hybridized to a second portion of the concatemer template molecule thereby forming a second binding complex, wherein the first and second binding complexes which are bound to the same multivalent molecule forms an avidity complex, and 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; **(b)** detecting the first and second binding complexes on the same concatemer template molecule; **(c)** imaging the optical color signals emitted from the detectably labeled multivalent molecule which forms the first and second binding complexes on the same concatemer template molecule; and **(d)** identifying the first nucleotide unit in the first binding complex thereby determining the sequence of the first portion of the concatemer template molecule, and identifying the second nucleotide unit in the second binding complex thereby determining the sequence of the second portion of the concatemer template molecule.

### Library Molecules

**[0521]** The present disclosure provides nucleic acid library molecules (100) and methods for preparing library-splint complexes (500) and covalently closed circular library molecules (600). The nucleic acid library molecules comprise DNA, RNA, cDNA or chimeric DNA/RNA. The nucleic acid library molecule can be single-stranded or double-stranded, or can include single-stranded or double-stranded portions. The nucleic acid library molecule can be linear, concatemeric, covalently closed circular, dumbbell, hairpin or other forms.

**[0522]** The nucleic acid library molecules described herein typically refers to a population of nucleic acid molecules each comprising a sequence of interest (e.g., insert (100)) covalently joined to at least one universal adaptor sequence (e.g., (120) and (130). Individual library molecules in the population can include additional universal adaptor sequences, and can further comprise at least one index sequence or unique identification sequence. Individual library molecules in the population can have a sequence of interest that is the same or different as other library molecules in the population.

**[0523]** The insert region of a nucleic acid library molecule comprises a sequence of interest extracted from any source including a biological sample (e.g., fresh or live sample) such as a single cell, a plurality of cells or tissue. The insert region can be isolated from healthy or diseases cells or tissues. The insert region can be obtained from an archived sample such as a fresh frozen paraffin embedded (FFPE) sample, or from needle biopsies, circulating tumor cells, cell free circulating DNA (e.g., from tumor cells or a fetus). Cells or tissues are typically treated with a lysis buffer to release their DNA and RNA, and the desired nucleic acid is separated from non-desired macromolecules such as proteins.

**[0524]** The insert region of a nucleic acid library molecule can be isolated in any form, including chromosomal, genomic (e.g., whole genomic), organellar (e.g., mitochondrial, chloroplast or ribosomal), recombinant molecules, cloned or amplified. The insert region of a nucleic acid library molecule can be methylated or non-methylated.

**[0525]** The insert region can be isolated from any organism including viruses, fungi, prokaryotes or eukaryotes. The insert region can be isolated from any organism including human, simian, ape, canine, feline, bovine, equine, murine, porcine, caprine, lupine, ranine, piscine, plant, insect or bacteria. The insert region can be isolated from organisms borne in air, water, soil or food.

**[0526]** The insert region can be isolated from any biological fluid, including blood, urine, serum, lymph, tumor, saliva, anal secretions, vaginal secretions, amniotic samples, perspiration, semen, environmental samples or culture samples. The insert region can be isolated from any organ, including head, neck, brain, breast, ovary, cervix, colon, rectum, endometrium, gallbladder, intestines, bladder, prostate, testicles, liver, lung, kidney, esophagus, pancreas, thyroid, pituitary, thymus, skin, heart, larynx, or other organs.

**[0527]** The insert region can be prepared using recombinant nucleic acid technology including but not limited to any combination of vector cloning, transgenic host cell preparation, host cell culturing and/or PCR amplification.

**[0528]** The insert region can be appended on one or both ends to at least one universal adaptor sequence to form a recombinant nucleic acid library molecule. The universal adaptor sequences can be prepared using chemical synthesis procedures using native nucleotides with or without nucleotide analogs or modified nucleotide linkages that confer certain properties, including resistance to enzymatic digestion, or increased thermal stability. Examples of nucleotide analogs and modified nucleotide linkages that inhibit nuclease digestion include phosphorothioate, 2'-O-methyl RNA, inverted dT, and 2' 3' dideoxy-dT. Insert regions that include locked nucleic acids (LNA) have increased thermal stability.

**[0529]** The insert region can be in fragmented or un-fragmented form. Fragmented insert regions can be obtained by mechanical force, enzymatic or chemical fragmentation methods. The fragmented insert regions can be generated using procedures that yield a population of fragments having overlapping sequences or non-overlapping sequences.

**[0530]** Mechanical fragmentation typically generates randomly fragmented nucleic acid molecules. Mechanical fragmentation methods include mechanical shearing such as fluid shear, constant shear and pulsatile shear. Mechanical fragmentation methods also include mechanical stress including sonication, nebulization and acoustic cavitation.

**[0531]** Enzymatic fragmentation procedures can be conducted under conditions suitable to generate randomly or non-randomly fragmented nucleic acid molecules. For example, restriction endonuclease enzyme digestion can be conducted to completion to generate non-randomly fragmented nucleic acid molecule. Alternatively, partial or incomplete restriction enzyme digestion can be conducted to generate randomly-fragmented nucleic acid molecules. Enzymatic fragmentation using restriction endonuclease enzymes includes any one or any combination of two or more restriction enzymes selected from a group consisting of type I, type II, type IIs, type IIB, type III, or type IV restriction enzymes. Enzymatic fragmentation includes digestion of the nucleic acid with a rare-cutting restriction enzyme, comprising Not I, Asc I, Bae I, AspC I, Pac I, Fse I, Sap I, Sfi I or Pst I. Enzymatic fragmentation include use of any combination of a nicking restriction endonuclease, endonuclease and/or exonuclease. Enzymatic fragmentation can be achieved by conducting a nick translation reaction.

**[0532]** Fragments of the insert region can be generated with PCR using sequence-specific primers that hybridize to target regions in genomic DNA samples to generate insert regions having known fragment lengths and sequences.

**[0533]** Targeted genome fragmentation methods using CRISPR/Cas9 can be used to generate fragmented insert regions.

**[0534]** Fragments of the insert portion can also be generated using a transposase-based tagmentation method using NEXTERA (from Epicentre).

**[0535]** The insert region can be single-stranded or double-stranded. The ends of the double-stranded insert region can be blunt-ended, or have a 5' overhang or a 3' overhang end, or any combination thereof. One or both ends of the insert region can be subjected to an enzymatic tailing reaction to generate a non-template poly-A tail by employing a terminal transferase reaction. The ends of the insert region can be compatible for joining to at least one universal adaptor sequence.

**[0536]** The insert region can be any length, for example the insert region can be about 50-250, or about 250-500, or about 500-750, or about 750-1000 bases or base pairs in length.

**[0537]** The fragments containing the insert region can be subjected to a size selection process, or the fragments are not size selected. For example, the fragments can be size selected by gel electrophoresis and gel slice extraction. The fragments can be size selected using a solid phase adherence/immobilization method which typically employs micro paramagnetic beads coated with a chemical functional group that interacts with nucleic acids under certain ionic strength conditions with or without polyethylene glycol or polyalkylene glycol. Commercially-available solid phase adherence beads include SPRI (Solid Phase Reversible Immobilization) beads from Beckman Coulter (AMPUR XP paramagnetic beads, catalog No. B23318), MAGNA PURE magnetic glass particles (Roche Diagnostics, catalog No. 03003990001), MAGNASIL paramagnetic beads from Promega (catalog No. MD1360), MAGTRATION paramagnetic beads and system from Precision System Science (catalog Nos. A1120 and A1060), MAG-BIND from Omega Bio-Tek (catalog No. M1378-01), MAGPREP silica from Millapore (catalog No. 101193), SNARE DNA purification systems from Bangs Laboratories (catalog Nos. BP691, BP692 and BP693), and CHEMAGEN M-PVA beads from Perkin Elmer (catalog No. CMG-200).

**[0538]** The insert region can be joined at one or both ends to at least one universal adaptor sequence using a ligase enzyme and/or primer extension reaction. Covalent linkage between an insert region and the universal adaptor(s) can be achieved with a DNA or RNA ligase. Exemplary DNA ligases that can ligate double-stranded DNA molecules include T4 DNA ligase and T7 DNA ligase. A universal adaptor sequence can be appended to an insert sequence by primer extension or PCR using a tailed primer having 5' region carrying a universal adaptor sequence and a 3' region that is complementary to a portion of the insert sequence. A universal adaptor sequence can be appended to an insert sequence which is flanked on one side or both sides with first and second universal adaptor sequences by primer

extension or PCR using a tailed primer having a 5' region carrying a third universal adaptor sequence and a 3' region that is complementary to a portion of the first or second universal adaptor sequence.

**[0539]** In some embodiments, a library molecule (100) can be generated by employing a ligation reaction and at least one primer extension reaction. The library molecule can be generated by **(a)** providing a double-stranded nucleic acid fragment comprising a sequence-of-interest (e.g., a double-stranded insert region (110)), **(b)** joining the first end of the double-stranded insert region (110) to a first double-stranded adaptor having a second left universal adaptor sequence (140), and joining the second end of the double-stranded insert region (110) to a second double-stranded adaptor having a second right universal adaptor sequence (150), wherein the joining is conducted using a DNA ligase enzyme to generate a double-stranded recombinant molecule. In some embodiments, the first and second double-stranded adaptors of step (b) comprise Y-shaped adaptors. In some embodiments, the Y-shaped adaptor comprises first and second oligonucleotide strands having partially complementary regions that hybridize together to form a double-stranded duplex region and a mismatched single-stranded region. In some embodiments, at least a portion of the first oligonucleotide strand comprises a second left universal adaptor sequence (140) or a complementary sequence thereof. In some embodiments, at least a portion of the second oligonucleotide strand comprises a second right universal adaptor sequence (150) or a complementary sequence thereof. The method for generating the library molecule further comprises **(c)** denaturing the double-stranded recombinant molecule of step (b) to generate single-stranded recombinant molecules, and **(d)** hybridizing the single-stranded recombinant molecules with a first tailed primer having a 5' region carrying a first left universal adaptor sequence (120) and a 3' region that is complementary to at least a portion of the second left universal adaptor sequence (140), and conducting a primer extension reaction thereby generating a double-stranded tailed molecule. In some embodiments, the first tailed primer of step (d) includes a first left index sequence (160). The method for generating a library molecule further comprises **(e)** hybridizing the double-stranded tailed molecule with a second tailed primer having a 5' region carrying a first right universal adaptor sequence (130) and a 3' region that is complementary to at least a portion of the second right universal adaptor sequence (150), and conducting a primer extension reaction thereby generating a library molecule comprising a first left universal adaptor sequence (120), a second left universal adaptor sequence (140), an insert region (110), a second right universal adaptor sequence (150), and a first right universal adaptor sequence (130). In some embodiments, the second tailed primer of step (e)

includes a first right index sequence (170). In some embodiments, a library molecule can be generated using ligation and primer extension reactions and conducting steps (a) – (e), with a PCR reaction. In some embodiments, a library molecule can be generated using ligation and primer extension reactions and conducting steps (a) – (e), with no PCR reaction, which reduces amplification bias. In some embodiments, in the method for generating a library molecule, the first left universal adaptor sequence (120) comprises a universal binding sequence for a first surface primer (or a complementary sequence thereof). In some embodiments, the first left universal adaptor sequence (120) in the library molecule can bind the first region of the first splint strand (320). In some embodiments, in the method for generating a library molecule, the first right universal adaptor sequence (130) comprises a universal binding sequence for a second surface primer (or a complementary sequence thereof). In some embodiments, the first right universal adaptor sequence (130) in the library molecule can bind the second region of the first splint strand (330). In some embodiments, in the method for generating a library molecule, the second left universal adaptor sequence (140) comprises a universal binding sequence for a first sequencing primer (or a complementary sequence thereof). In some embodiments, in the method for generating a library molecule, the second right universal adaptor sequence (150) comprises a universal binding sequence for a second sequencing primer (or a complementary sequence thereof).

**[0540]** In some embodiments, the method for generating a library molecule further comprises appending additional universal adaptor sequences to the library molecule (100) (e.g., FIGS. 4, 5, 6 or 7) by conducting at least one primer extension reaction or PCR using additional tailed primers. In some embodiments, the appending methods that employ the additional tailed primers can be used to append additional universal adaptor sequences to the library molecule, or to replace at least a portion of a universal adaptor sequence in a library molecule. In some embodiments, the additional tailed primers comprise a 5' region carrying a first additional universal adaptor sequence and a 3' region that is complementary to at least a portion of the first left universal adaptor sequence (120), wherein the first additional universal adaptor sequence in the additional tailed primers comprise a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer and/or a universal binding sequence for a compaction oligonucleotide. In some embodiments, the additional tailed primers comprise a 5' region carrying a second additional universal adaptor sequence and a 3' region that is complementary to at least a portion of the first right universal adaptor sequence (130), wherein the second additional universal adaptor sequence

in the additional tailed primers comprise a universal binding sequence for a forward or reverse sequencing primer, a universal binding sequence for a first or second surface primer, a universal binding sequence for a forward or reverse amplification primer and/or a universal binding sequence for a compaction oligonucleotide.

**[0541]** In some embodiments, methods for appending additional universal adaptor sequences to nucleic acid library molecules comprise: **step (a)** providing a plurality of nucleic acid library molecules (100) each comprising sequences arranged in a 5' to 3' order: (i) a first left universal adaptor sequence (120), (ii) a second left universal adaptor sequence (140), (iii) a sequence of interest (110), (iv) a second right universal adaptor sequence (150), and (v) a first right universal adaptor sequence (130); **step (b)** hybridizing individual library molecules of step (a) with a first tailed primer and conducting a first primer extension reaction (or first PCR), wherein the first tailed primers comprise a 5' region carrying a third left universal adaptor sequence and a 3' region that is complementary to at least a portion of the first left universal adaptor sequence (120) of the library molecule (100), wherein the first primer extension reaction (or first PCR) generates a plurality of first modified library molecules each appended at one end with the third left universal adaptor sequence, wherein the third left universal adaptor sequence of the first tailed primers comprise (i) a universal binding sequence for a forward or reverse sequencing primer, (ii) a universal binding sequence for a first or second surface primer, (iii) a universal binding sequence for a forward or reverse amplification primer and/or (iv) a universal binding sequence for a compaction oligonucleotide; and **step (c)** hybridizing individual first modified library molecules of step (a) with a second tailed primer and conducting a second primer extension reaction (or second PCR), wherein the second tailed primers comprise a 5' region carrying a third right universal adaptor sequence and a 3' region that is complementary to at least a portion of the first right universal adaptor sequence (130) of the first modified library molecule, wherein the second primer extension reaction (or second PCR) generates a plurality of second modified library molecules each appended at one end with the third right universal adaptor sequence, wherein the third right universal adaptor sequence of the second tailed primers comprise (i) a universal binding sequence for a forward or reverse sequencing primer, (ii) a universal binding sequence for a first or second surface primer, (iii) a universal binding sequence for a forward or reverse amplification primer and/or (iv) a universal binding sequence for a compaction oligonucleotide.

**[0542]** In some embodiments, the first primer extension reaction (or first PCR) of step (b) appends a full-length or a portion of the third left universal adaptor sequence to one end of

the first modified library molecules. In some embodiments, the second primer extension reaction (or second PCR) of step (c) appends a full-length or a portion of the third right universal adaptor sequence to one end of the second modified library molecules.

**[0543]** In some embodiments, the nucleic acid library molecules of step (a) each comprise sequences arranged in a 5' to 3' order: (i) a first left universal adaptor sequence (120) having a binding sequence for a first surface primer, (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.

**[0544]** In some embodiments, methods for appending *more* additional universal adaptor sequences to nucleic acid library molecules comprise: **step (d)** hybridizing individual second modified library molecules of step (c) with a third tailed primer and conducting a third primer extension reaction (or third PCR), wherein the third tailed primers comprise a 5' region carrying a fourth left universal adaptor sequence and a 3' region that is complementary to at least a portion of the third left universal adaptor sequence of the second modified library molecule, wherein the third primer extension reaction (or third PCR) generates a plurality of third modified library molecules each appended at one end with the fourth left universal adaptor sequence, wherein the fourth left universal adaptor sequence of the third tailed primers comprise (i) a universal binding sequence for a forward or reverse sequencing primer, (ii) a universal binding sequence for a first or second surface primer, (iii) a universal binding sequence for a forward or reverse amplification primer and/or (iv) a universal binding sequence for a compaction oligonucleotide; and **step (e)** hybridizing individual third modified library molecules of step (d) with a fourth tailed primer and conducting a fourth primer extension reaction (or fourth PCR), wherein the fourth tailed primers comprise a 5' region carrying a fourth right universal adaptor sequence and a 3' region that is complementary to at least a portion of the third right universal adaptor sequence of the third modified library molecule, wherein the fourth primer extension reaction (or fourth PCR) generates a plurality of fourth modified library molecules each appended at one end with the fourth right universal adaptor sequence, wherein the fourth right universal adaptor sequence of the fourth tailed primers comprise (i) a universal binding sequence for a forward or reverse sequencing primer, (ii) a universal binding sequence for a first or second surface primer, (iii) a universal binding sequence for a forward or reverse amplification primer and/or (iv) a universal binding sequence for a compaction oligonucleotide.

[0545] In some embodiments, the third primer extension reaction (or third PCR) of step (d) appends a full-length or a portion of the fourth left universal adaptor sequence to one end of the third modified library molecules. In some embodiments, the fourth primer extension reaction (or fourth PCR) of step (e) appends a full-length or a portion of the fourth right universal adaptor sequence to one end of the fourth modified library molecules.

[0546] In some embodiments, methods for appending *even more* additional universal adaptor sequences to nucleic acid library molecules comprise: **step (f)** hybridizing individual fourth modified library molecules of step (e) with a fifth tailed primer and conducting a fifth primer extension reaction (or fifth PCR), wherein the fifth tailed primers comprise a 5' region carrying a fifth left universal adaptor sequence and a 3' region that is complementary to at least a portion of the fourth left universal adaptor sequence of the fourth modified library molecule, wherein the fifth primer extension reaction (or fifth PCR) generates a plurality of fifth modified library molecules each appended at one end with the fifth left universal adaptor sequence, wherein the fifth left universal adaptor sequence of the fifth tailed primers comprise (i) a universal binding sequence for a forward or reverse sequencing primer, (ii) a universal binding sequence for a first or second surface primer, (iii) a universal binding sequence for a forward or reverse amplification primer and/or (iv) a universal binding sequence for a compaction oligonucleotide; and **step (g)** hybridizing individual fifth modified library molecules of step (f) with a sixth tailed primer and conducting a sixth primer extension reaction (or sixth PCR), wherein the sixth tailed primers comprise a 5' region carrying a sixth right universal adaptor sequence and a 3' region that is complementary to at least a portion of the fourth right universal adaptor sequence of the fifth modified library molecule, wherein the sixth primer extension reaction (or sixth PCR) generates a plurality of sixth modified library molecules each appended at one end with the sixth right universal adaptor sequence, wherein the sixth right universal adaptor sequence of the sixth tailed primers comprise (i) a universal binding sequence for a forward or reverse sequencing primer, (ii) a universal binding sequence for a first or second surface primer, (iii) a universal binding sequence for a forward or reverse amplification primer and/or (iv) a universal binding sequence for a compaction oligonucleotide.

[0547] In some embodiments, the fifth primer extension reaction (or fifth PCR) of step (f) appends a full-length or a portion of the fifth left universal adaptor sequence to one end of the fifth modified library molecules. In some embodiments, the sixth primer extension reaction (or sixth PCR) of step (g) appends a full-length or a portion of the fifth right universal adaptor sequence to one end of the sixth modified library molecules.

[0548] In some embodiments, the first tailed primer of step (b), the third tailed primer of step (d) and/or the fifth tailed primer of step (f) further comprise a left index sequence.

[0549] In some embodiments, the first tailed primer of step (b), the third tailed primer of step (d) and/or the fifth tailed primer of step (f) further comprise a left unique identification sequence.

[0550] In some embodiments, the second tailed primer of step (c), the fourth tailed primer of step (e) and/or the sixth tailed primer of step (g) further comprises a right index sequence.

[0551] In some embodiments, the second tailed primer of step (c), the fourth tailed primer of step (e) and/or the sixth tailed primer of step (g) further comprises a right unique identification sequence.

[0552] In some embodiments, the appended third left universal adaptor sequence of step (b) can bind a first region (320) of a first splint strand.

[0553] In some embodiments, the appended third right universal adaptor sequence of step (c) can bind a second region (330) of a first splint strand.

[0554] In some embodiments, the appended fourth left universal adaptor sequence of step (d) can bind a first region (320) of a first splint strand.

[0555] In some embodiments, the appended fourth right universal adaptor sequence of step (e) can bind a second region (330) of a first splint strand.

[0556] In some embodiments, the appended fifth left universal adaptor sequence of step (f) can bind a first region (320) of a first splint strand.

[0557] In some embodiments, the appended sixth right universal adaptor sequence of step (g) can bind a second region (330) of a first splint strand.

[0558] The skilled artisan will appreciate that additional universal adaptor sequences can be appended to the library molecule by repeating the primer extension reactions or PCR. In some embodiments, the library molecule can be appended with ten or more additional universal adaptor sequences on one or both sides of the library molecule.

### **Supports with Low Non-Specific Binding Coatings**

[0559] The present disclosure provides compositions and methods for sequencing which employ a support having a plurality of surface primers immobilized thereon. In some embodiments, the support is passivated with a low non-specific binding coating. The surface coatings described herein exhibit very low non-specific binding to reagents typically used for nucleic acid capture, amplification and sequencing workflows, such as dyes, nucleotides, enzymes, and nucleic acid primers. The surface coatings exhibit low background

fluorescence signals or high contrast-to-noise (CNR) ratios compared to conventional surface coatings.

**[0560]** In general, the supports comprise a substrate (or support structure), one or more layers of a covalently or non-covalently attached low-binding, chemical modification layers, e.g., silane layers, polymer films, and one or more covalently or non-covalently attached primer sequences that may be used for tethering single-stranded target nucleic acid(s) to the support surface. In some embodiments, the formulation of the surface, e.g., the chemical composition of one or more layers, the coupling chemistry used to cross-link the one or more layers to the support surface and/or to each other, and the total number of layers, may be varied such that non-specific binding of proteins, nucleic acid molecules, and other hybridization and amplification reaction components to the support surface is minimized or reduced relative to a comparable monolayer. Often, the formulation of the surface may be varied such that non-specific hybridization on the support surface is minimized or reduced relative to a comparable monolayer. The formulation of the surface may be varied such that non-specific amplification on the support surface is minimized or reduced relative to a comparable monolayer. The formulation of the surface may be varied such that specific amplification rates and/or yields on the support surface are maximized. Amplification levels suitable for detection are achieved in no more than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, or more than 30 amplification cycles in some cases disclosed herein.

**[0561]** The substrate or support structure that comprises the one or more chemically-modified layers, e.g., layers of a low non-specific binding polymer, may be independent or integrated into another structure or assembly. For example, in some embodiments, the substrate or support structure may comprise one or more surfaces within an integrated or assembled microfluidic flow cell. The substrate or support structure may comprise one or more surfaces within a microplate format, e.g., the bottom surface of the wells in a microplate. As noted above, in some preferred embodiments, the substrate or support structure comprises the interior surface (such as the lumen surface) of a capillary. In alternate preferred embodiments the substrate or support structure comprises the interior surface (such as the lumen surface) of a capillary etched into a planar chip.

**[0562]** The attachment chemistry used to graft a first chemically-modified layer to a surface will generally be dependent on both the material from which the surface is fabricated and the chemical nature of the layer. In some embodiments, the first layer may be covalently attached to the surface. In some embodiments, the first layer may be non-covalently attached, e.g., adsorbed to the surface through non-covalent interactions such as electrostatic

interactions, hydrogen bonding, or van der Waals interactions between the surface and the molecular components of the first layer. In either case, the substrate surface may be treated prior to attachment or deposition of the first layer. Any of a variety of surface preparation techniques known to those of skill in the art may be used to clean or treat the surface. For example, glass or silicon surfaces may be acid-washed using a Piranha solution (a mixture of sulfuric acid ( $\text{H}_2\text{SO}_4$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ )), base treatment in KOH and NaOH, and/or cleaned using an oxygen plasma treatment method.

**[0563]** Silane chemistries constitute one non-limiting approach for covalently modifying the silanol groups on glass or silicon surfaces to attach more reactive functional groups (e.g., amines or carboxyl groups), which may then be used in coupling linker molecules (e.g., linear hydrocarbon molecules of various lengths, such as C6, C12, C18 hydrocarbons, or linear polyethylene glycol (PEG) molecules) or layer molecules (e.g., branched PEG molecules or other polymers) to the surface. Examples of suitable silanes that may be used in creating any of the disclosed low binding surfaces include, but are not limited to, (3-Aminopropyl) trimethoxysilane (APTMS), (3-Aminopropyl) triethoxysilane (APTES), any of a variety of PEG-silanes (e.g., comprising molecular weights of 1K, 2K, 5K, 10K, 20K, etc.), amino-PEG silane (i.e., comprising a free amino functional group), maleimide-PEG silane, biotin-PEG silane, and the like.

**[0564]** Any of a variety of molecules known to those of skill in the art including, but not limited to, amino acids, peptides, nucleotides, oligonucleotides, other monomers or polymers, or combinations thereof may be used in creating the one or more chemically-modified layers on the surface, where the choice of components used may be varied to alter one or more properties of the surface, e.g., the surface density of functional groups and/or tethered oligonucleotide primers, the hydrophilicity/hydrophobicity of the surface, or the three-dimensional nature (i.e., “thickness”) of the surface. Examples of preferred polymers that may be used to create one or more layers of low non-specific binding material in any of the disclosed surfaces include, but are not limited to, polyethylene glycol (PEG) of various molecular weights and branching structures, streptavidin, polyacrylamide, polyester, dextran, poly-lysine, and poly-lysine copolymers, or any combination thereof. Examples of conjugation chemistries that may be used to graft one or more layers of material (e.g. polymer layers) to the surface and/or to cross-link the layers to each other include, but are not limited to, biotin-streptavidin interactions (or variations thereof), his tag – Ni/NTA conjugation chemistries, methoxy ether conjugation chemistries, carboxylate conjugation

chemistries, amine conjugation chemistries, NHS esters, maleimides, thiol, epoxy, azide, hydrazide, alkyne, isocyanate, and silane.

**[0565]** The low non-specific binding surface coating may be applied uniformly across the substrate. Alternately, the surface coating may be patterned, such that the chemical modification layers are confined to one or more discrete regions of the substrate. For example, the surface may be patterned using photolithographic techniques to create an ordered array or random pattern of chemically-modified regions on the surface. Alternately or in combination, the substrate surface may be patterned using, e.g., contact printing and/or ink-jet printing techniques. In some embodiments, an ordered array or random pattern of chemically-modified regions may comprise at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, or 10,000 or more discrete regions.

**[0566]** In order to achieve low nonspecific binding surfaces, hydrophilic polymers may be nonspecifically adsorbed or covalently grafted to the surface. Typically, passivation is performed utilizing poly(ethylene glycol) (PEG, also known as polyethylene oxide (PEO) or polyoxyethylene) or other hydrophilic polymers with different molecular weights and end groups that are linked to a surface using, for example, silane chemistry. The end groups distal from the surface can include, but are not limited to, biotin, methoxy ether, carboxylate, amine, NHS ester, maleimide, and bis-silane. In some embodiments, two or more layers of a hydrophilic polymer, e.g., a linear polymer, branched polymer, or multi-branched polymer, may be deposited on the surface. In some embodiments, two or more layers may be covalently coupled to each other or internally cross-linked to improve the stability of the resulting surface. In some embodiments, oligonucleotide primers with different base sequences and base modifications (or other biomolecules, e.g., enzymes or antibodies) may be tethered to the resulting surface layer at various surface densities. In some embodiments, for example, both surface functional group density and oligonucleotide concentration may be varied to target a certain primer density range. Additionally, primer density can be controlled by diluting oligonucleotide with other molecules that carry the same functional group. For example, amine-labeled oligonucleotide can be diluted with amine-labeled polyethylene glycol in a reaction with an NHS-ester coated surface to reduce the final primer density. Primers with different lengths of linker between the hybridization region and the surface attachment functional group can also be applied to control surface density. Example of suitable linkers include poly-T and poly-A strands at the 5' end of the primer (e.g., 0 to 20 bases), PEG linkers (e.g., 3 to 20 monomer units), and carbon-chain (e.g., C6, C12, C18,

etc.). To measure the primer density, fluorescently-labeled primers may be tethered to the surface and a fluorescence reading then compared with that for a dye solution of known concentration.

**[0567]** In order to scale primer surface density and add additional dimensionality to hydrophilic or amphoteric surfaces, surfaces comprising multi-layer coatings of PEG and other hydrophilic polymers have been developed. By using hydrophilic and amphoteric surface layering approaches that include, but are not limited to, the polymer/co-polymer materials described below, it is possible to increase primer loading density on the surface significantly. Traditional PEG coating approaches use monolayer primer deposition, which have been generally reported for single molecule applications, but do not yield high copy numbers for nucleic acid amplification applications. As described herein “layering” can be accomplished using traditional crosslinking approaches with any compatible polymer or monomer subunits such that a surface comprising two or more highly crosslinked layers can be built sequentially. Examples of suitable polymers include, but are not limited to, streptavidin, poly acrylamide, polyester, dextran, poly-lysine, and copolymers of poly-lysine and PEG. In some embodiments, the different layers may be attached to each other through any of a variety of conjugation reactions including, but not limited to, biotin-streptavidin binding, azide-alkyne click reaction, amine-NHS ester reaction, thiol-maleimide reaction, and ionic interactions between positively charged polymer and negatively charged polymer. In some embodiments, high primer density materials may be constructed in solution and subsequently layered onto the surface in multiple steps.

**[0568]** As noted, the low non-specific binding coatings of the present disclosure exhibit reduced non-specific binding of proteins, nucleic acids, and other components of the hybridization and/or amplification formulation used for solid-phase nucleic acid amplification. The degree of non-specific binding exhibited by a given support surface may be assessed either qualitatively or quantitatively. For example, in some embodiments, exposure of the surface to fluorescent dyes (e.g., cyanine dyes such as Cy3, or Cy5, etc., fluoresceins, coumarins, rhodamines, etc. or other dyes disclosed herein), fluorescently-labeled nucleotides, fluorescently-labeled oligonucleotides, and/or fluorescently-labeled proteins (e.g. polymerases) under a standardized set of conditions, followed by a specified rinse protocol and fluorescence imaging may be used as a qualitative tool for comparison of non-specific binding on supports comprising different surface formulations. In some embodiments, exposure of the surface to fluorescent dyes, fluorescently-labeled nucleotides, fluorescently-labeled oligonucleotides, and/or fluorescently-labeled proteins (e.g.

polymerases) under a standardized set of conditions, followed by a specified rinse protocol and fluorescence imaging may be used as a quantitative tool for comparison of non-specific binding on supports comprising different surface formulations – provided that care has been taken to ensure that the fluorescence imaging is performed under a condition where fluorescence signal is linearly related (or related in a predictable manner) to the number of fluorophores on the support surface (e.g., under a condition where signal saturation and/or self-quenching of the fluorophore is not an issue) and suitable calibration standards are used. In some embodiments, other techniques known to those of skill in the art, for example, radioisotope labeling and counting methods may be used for quantitative assessment of the degree to which non-specific binding is exhibited by the different support surface formulations of the present disclosure.

**[0569]** Some surfaces disclosed herein exhibit a ratio of specific to nonspecific binding of a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100, or greater than 100, or any intermediate value spanned by the range herein. Some surfaces disclosed herein exhibit a ratio of specific to nonspecific fluorescence of a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100, or greater than 100, or any intermediate value spanned by the range herein.

**[0570]** As noted, in some embodiments, the degree of non-specific binding exhibited by the disclosed low-binding supports may be assessed using a standardized protocol for contacting the surface with a labeled protein (e.g., bovine serum albumin (BSA), streptavidin, a DNA polymerase, a reverse transcriptase, a helicase, a single-stranded binding protein (SSB), etc., or any combination thereof), a labeled nucleotide, a labeled oligonucleotide, etc., under a standardized set of incubation and rinse conditions, followed by detection of the amount of label remaining on the surface and comparison of the signal resulting therefrom to an appropriate calibration standard. In some embodiments, the label may comprise a fluorescent label. In some embodiments, the label may comprise a radioisotope. In some embodiments, the label may comprise any other detectable label known to one of skill in the art. In some embodiments, the degree of non-specific binding exhibited by a given support surface formulation may thus be assessed in terms of the number of non-specifically bound protein molecules (or other molecules) per unit area. In some embodiments, the low-binding supports of the present disclosure may exhibit non-specific protein binding (or non-specific binding of other specified molecules, (e.g., cyanine dyes such as Cy3, or Cy5, etc., fluoresceins, coumarins, rhodamines, etc. or other dyes disclosed herein)) of less than 0.001

molecule per  $\mu\text{m}^2$ , less than 0.01 molecule per  $\mu\text{m}^2$ , less than 0.1 molecule per  $\mu\text{m}^2$ , less than 0.25 molecule per  $\mu\text{m}^2$ , less than 0.5 molecule per  $\mu\text{m}^2$ , less than 1 molecule per  $\mu\text{m}^2$ , less than 10 molecules per  $\mu\text{m}^2$ , less than 100 molecules per  $\mu\text{m}^2$ , or less than 1,000 molecules per  $\mu\text{m}^2$ . Those of skill in the art will realize that a given support surface of the present disclosure may exhibit non-specific binding falling anywhere within this range, for example, of less than 86 molecules per  $\mu\text{m}^2$ . For example, some modified surfaces disclosed herein exhibit nonspecific protein binding of less than 0.5 molecule /  $\mu\text{m}^2$  following contact with a 1  $\mu\text{M}$  solution of Cy3 labeled streptavidin (GE Amersham) in phosphate buffered saline (PBS) buffer for 15 minutes, followed by 3 rinses with deionized water. Some modified surfaces disclosed herein exhibit nonspecific binding of Cy3 dye molecules of less than 0.25 molecules per  $\mu\text{m}^2$ . In independent nonspecific binding assays, 1  $\mu\text{M}$  labeled Cy3 SA (ThermoFisher), 1  $\mu\text{M}$  Cy5 SA dye (ThermoFisher), 10  $\mu\text{M}$  Aminoallyl-dUTP - ATTO-647N (Jena Biosciences), 10  $\mu\text{M}$  Aminoallyl-dUTP - ATTO-Rho11 (Jena Biosciences), 10  $\mu\text{M}$  Aminoallyl-dUTP - ATTO-Rho11 (Jena Biosciences), 10  $\mu\text{M}$  7-Propargylamino-7-deaza-dGTP - Cy5 (Jena Biosciences, and 10  $\mu\text{M}$  7-Propargylamino-7-deaza-dGTP - Cy3 (Jena Biosciences) were incubated on the low binding substrates at 37°C for 15 minutes in a 384 well plate format. Each well was rinsed 2-3 x with 50  $\mu\text{l}$  deionized RNase/DNase Free water and 2-3 x with 25 mM ACES buffer pH 7.4. The 384 well plates were imaged on a GE Typhoon instrument using the Cy3, AF555, or Cy5 filter sets (according to dye test performed) as specified by the manufacturer at a PMT gain setting of 800 and resolution of 50-100  $\mu\text{m}$ . For higher resolution imaging, images were collected on an Olympus IX83 microscope (Olympus Corp., Center Valley, PA) with a total internal reflectance fluorescence (TIRF) objective (100X, 1.5 NA, Olympus), a CCD camera (e.g., an Olympus EM-CCD monochrome camera, Olympus XM-10 monochrome camera, or an Olympus DP80 color and monochrome camera), an illumination source (e.g., an Olympus 100W Hg lamp, an Olympus 75W Xe lamp, or an Olympus U-HGLGPS fluorescence light source), and excitation wavelengths of 532 nm or 635 nm. Dichroic mirrors were purchased from Semrock (IDEX Health & Science, LLC, Rochester, New York), e.g., 405, 488, 532, or 633 nm dichroic reflectors/beamsplitters, and band pass filters were chosen as 532 LP or 645 LP concordant with the appropriate excitation wavelength. Some modified surfaces disclosed herein exhibit nonspecific binding of dye molecules of less than 0.25 molecules per  $\mu\text{m}^2$ .

**[0571]** In some embodiments, the surfaces disclosed herein exhibit a ratio of specific to nonspecific binding of a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100, or greater than 100, or any

intermediate value spanned by the range herein. In some embodiments, the surfaces disclosed herein exhibit a ratio of specific to nonspecific fluorescence signals for a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100, or greater than 100, or any intermediate value spanned by the range herein.

**[0572]** The low-background surfaces consistent with the disclosure herein may exhibit specific dye attachment (e.g., Cy3 attachment) to non-specific dye adsorption (e.g., Cy3 dye adsorption) ratios of at least 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1, 20:1, 30:1, 40:1, 50:1, or more than 50 specific dye molecules attached per molecule nonspecifically adsorbed. Similarly, when subjected to an excitation energy, low-background surfaces consistent with the disclosure herein to which fluorophores, e.g., Cy3, have been attached may exhibit ratios of specific fluorescence signal (e.g., arising from Cy3-labeled oligonucleotides attached to the surface) to non-specific adsorbed dye fluorescence signals of at least 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1, 20:1, 30:1, 40:1, 50:1, or more than 50:1.

**[0573]** In some embodiments, the degree of hydrophilicity (or “wettability” with aqueous solutions) of the disclosed support surfaces may be assessed, for example, through the measurement of water contact angles in which a small droplet of water is placed on the surface and its angle of contact with the surface is measured using, e.g., an optical tensiometer. In some embodiments, a static contact angle may be determined. In some embodiments, an advancing or receding contact angle may be determined. In some embodiments, the water contact angle for the hydrophilic, low-binding support surfaced disclosed herein may range from about 0 degrees to about 30 degrees. In some embodiments, the water contact angle for the hydrophilic, low-binding support surfaced disclosed herein may no more than 50 degrees, 40 degrees, 30 degrees, 25 degrees, 20 degrees, 18 degrees, 16 degrees, 14 degrees, 12 degrees, 10 degrees, 8 degrees, 6 degrees, 4 degrees, 2 degrees, or 1 degree. In many cases the contact angle is no more than 40 degrees. Those of skill in the art will realize that a given hydrophilic, low-binding support surface of the present disclosure may exhibit a water contact angle having a value of anywhere within this range.

**[0574]** In some embodiments, the hydrophilic surfaces disclosed herein facilitate reduced wash times for bioassays, often due to reduced nonspecific binding of biomolecules to the low-binding surfaces. In some embodiments, adequate wash steps may be performed in less than 60, 50, 40, 30, 20, 15, 10, or less than 10 seconds. For example, in some embodiments adequate wash steps may be performed in less than 30 seconds.

**[0575]** The low-binding surfaces of the present disclosure exhibit significant improvement in stability or durability to prolonged exposure to solvents and elevated temperatures, or to repeated cycles of solvent exposure or changes in temperature. For example, in some embodiments, the stability of the disclosed surfaces may be tested by fluorescently labeling a functional group on the surface, or a tethered biomolecule (e.g., an oligonucleotide primer) on the surface, and monitoring fluorescence signal before, during, and after prolonged exposure to solvents and elevated temperatures, or to repeated cycles of solvent exposure or changes in temperature. In some embodiments, the degree of change in the fluorescence used to assess the quality of the surface may be less than 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, or 25% over a time period of 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 60 minutes, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 15 hours, 20 hours, 25 hours, 30 hours, 35 hours, 40 hours, 45 hours, 50 hours, or 100 hours of exposure to solvents and/or elevated temperatures (or any combination of these percentages as measured over these time periods). In some embodiments, the degree of change in the fluorescence used to assess the quality of the surface may be less than 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, or 25% over 5 cycles, 10 cycles, 20 cycles, 30 cycles, 40 cycles, 50 cycles, 60 cycles, 70 cycles, 80 cycles, 90 cycles, 100 cycles, 200 cycles, 300 cycles, 400 cycles, 500 cycles, 600 cycles, 700 cycles, 800 cycles, 900 cycles, or 1,000 cycles of repeated exposure to solvent changes and/or changes in temperature (or any combination of these percentages as measured over this range of cycles).

**[0576]** In some embodiments, the surfaces disclosed herein may exhibit a high ratio of specific signal to nonspecific signal or other background. For example, when used for nucleic acid amplification, some surfaces may exhibit an amplification signal that is at least 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, 100, or greater than 100 fold greater than a signal of an adjacent unpopulated region of the surface. Similarly, some surfaces exhibit an amplification signal that is at least 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, 100, or greater than 100 fold greater than a signal of an adjacent amplified nucleic acid population region of the surface.

**[0577]** In some embodiments, fluorescence images of the disclosed low background surfaces when used in nucleic acid hybridization or amplification applications to create clusters of hybridized or clonally-amplified nucleic acid molecules (e.g., that have been directly or indirectly labeled with a fluorophore) exhibit contrast-to-noise ratios (CNRs) of at

least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, or greater than 250.

**[0578]** One or more types of primer (e.g., capture oligonucleotides and/or circularization oligonucleotides) may be attached or tethered to the support surface. In some embodiments, the one or more types of adaptors or primers may comprise spacer sequences, adaptor sequences for hybridization to adaptor-ligated target library nucleic acid sequences, forward amplification primers, reverse amplification primers, sequencing primers, and/or molecular barcoding sequences, or any combination thereof. In some embodiments, 1 primer or adaptor sequence may be tethered to at least one layer of the surface. In some embodiments, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 different primer or adaptor sequences may be tethered to at least one layer of the surface.

**[0579]** In some embodiments, the tethered adaptor and/or primer sequences may range in length from about 10 nucleotides to about 100 nucleotides. In some embodiments, the tethered adaptor and/or primer sequences may be at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, or at least 100 nucleotides in length. In some embodiments, the tethered adaptor and/or primer sequences may be at most 100, at most 90, at most 80, at most 70, at most 60, at most 50, at most 40, at most 30, at most 20, or at most 10 nucleotides in length. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some embodiments the length of the tethered adaptor and/or primer sequences may range from about 20 nucleotides to about 80 nucleotides. Those of skill in the art will recognize that the length of the tethered adaptor and/or primer sequences may have any value within this range, e.g., about 24 nucleotides.

**[0580]** In some embodiments, the resultant surface density of primers on the low binding support surfaces of the present disclosure may range from about 100 primer molecules per  $\mu\text{m}^2$  to about 100,000 primer molecules per  $\mu\text{m}^2$ . In some embodiments, the resultant surface density of primers on the low binding support surfaces of the present disclosure may range from about 100,000 primer molecules per  $\mu\text{m}^2$  to about  $10^{15}$  primer molecules per  $\mu\text{m}^2$ . In some embodiments, the surface density of primers may be at least 1,000, at least 10,000, at least 100,000, or at least  $10^{15}$  primer molecules per  $\mu\text{m}^2$ . In some embodiments, the surface density of primers may be at most 10,000, at most 100,000, at most 1,000,000, or at most  $10^{15}$  primer molecules per  $\mu\text{m}^2$ . Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some embodiments the surface density of primers may range from about 10,000 molecules

per  $\mu\text{m}^2$  to about  $10^{15}$  molecules per  $\mu\text{m}^2$ . Those of skill in the art will recognize that the surface density of primer molecules may have any value within this range, e.g., about 455,000 molecules per  $\mu\text{m}^2$ . In some embodiments, the surface density of target library nucleic acid sequences initially hybridized to adaptor or primer sequences on the support surface may be less than or equal to that indicated for the surface density of tethered primers. In some embodiments, the surface density of clonally-amplified target library nucleic acid sequences hybridized to adaptor or primer sequences on the support surface may span the same range as that indicated for the surface density of tethered primers.

**[0581]** Local densities as listed above do not preclude variation in density across a surface, such that a surface may comprise a region having an oligo density of, for example, 500,000 per  $\mu\text{m}^2$ , while also comprising at least a second region having a substantially different local density.

**[0582]** The low non-specific binding coating comprise one or more layers of a multi-layered surface coating may comprise a branched polymer or may be linear. Examples of suitable branched polymers include, but are not limited to, branched PEG, branched poly(vinyl alcohol) (branched PVA), branched poly(vinyl pyridine), branched poly(vinyl pyrrolidone) (branched PVP), branched ), poly(acrylic acid) (branched PAA), branched polyacrylamide, branched poly(N-isopropylacrylamide) (branched PNIPAM), branched poly(methyl methacrylate) (branched PMA), branched poly(2-hydroxyethyl methacrylate) (branched PHEMA), branched poly(oligo(ethylene glycol) methyl ether methacrylate) (branched POEGMA), branched polyglutamic acid (branched PGA), branched poly-lysine, branched poly-glucoside, and dextran.

**[0583]** In some embodiments, the branched polymers used to create one or more layers of any of the multi-layered surfaces disclosed herein may comprise at least 4 branches, at least 5 branches, at least 6 branches, at least 7 branches, at least 8 branches, at least 9 branches, at least 10 branches, at least 12 branches, at least 14 branches, at least 16 branches, at least 18 branches, at least 20 branches, at least 22 branches, at least 24 branches, at least 26 branches, at least 28 branches, at least 30 branches, at least 32 branches, at least 34 branches, at least 36 branches, at least 38 branches, or at least 40 branched.

**[0584]** Linear, branched, or multi-branched polymers used to create one or more layers of any of the multi-layered surfaces disclosed herein may have a molecular weight of at least 500, at least 1,000, at least 2,000, at least 3,000, at least 4,000, at least 5,000, at least 10,000, at least 15,000, at least 20,000, at least 25,000, at least 30,000, at least 35,000, at least 40,000, at least 45,000, or at least 50,000 daltons.

**[0585]** In some embodiments, e.g., wherein at least one layer of a multi-layered surface comprises a branched polymer, the number of covalent bonds between a branched polymer molecule of the layer being deposited and molecules of the previous layer may range from about one covalent linkage per molecule to about 32 covalent linkages per molecule. In some embodiments, the number of covalent bonds between a branched polymer molecule of the new layer and molecules of the previous layer may be at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 12, at least 14, at least 16, at least 18, at least 20, at least 22, at least 24, at least 26, at least 28, at least 30, or at least 32 covalent linkages per molecule.

**[0586]** Any reactive functional groups that remain following the coupling of a material layer to the surface may optionally be blocked by coupling a small, inert molecule using a high yield coupling chemistry. For example, in the case that amine coupling chemistry is used to attach a new material layer to the previous one, any residual amine groups may subsequently be acetylated or deactivated by coupling with a small amino acid such as glycine.

**[0587]** The number of layers of low non-specific binding material, e.g., a hydrophilic polymer material, deposited on the surface, may range from 1 to about 10. In some embodiments, the number of layers is at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10. In some embodiments, the number of layers may be at most 10, at most 9, at most 8, at most 7, at most 6, at most 5, at most 4, at most 3, at most 2, or at most 1. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some embodiments the number of layers may range from about 2 to about 4. In some embodiments, all of the layers may comprise the same material. In some embodiments, each layer may comprise a different material. In some embodiments, the plurality of layers may comprise a plurality of materials. In some embodiments at least one layer may comprise a branched polymer. In some embodiment, all of the layers may comprise a branched polymer.

**[0588]** One or more layers of low non-specific binding material may in some cases be deposited on and/or conjugated to the substrate surface using a polar protic solvent, a polar or polar aprotic solvent, a nonpolar solvent, or any combination thereof. In some embodiments the solvent used for layer deposition and/or coupling may comprise an alcohol (e.g., methanol, ethanol, propanol, etc.), another organic solvent (e.g., acetonitrile, dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), etc.), water, an aqueous buffer solution

(e.g., phosphate buffer, phosphate buffered saline, 3-(N-morpholino)propanesulfonic acid (MOPS), etc.), or any combination thereof. In some embodiments, an organic component of the solvent mixture used may comprise at least 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% of the total, with the balance made up of water or an aqueous buffer solution. In some embodiments, an aqueous component of the solvent mixture used may comprise at least 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% of the total, with the balance made up of an organic solvent. The pH of the solvent mixture used may be less than 6, about 6, 6.5, 7, 7.5, 8, 8.5, 9, or greater than pH 9.

**[0589]** Fluorescence imaging may be performed using any of a variety of fluorophores, fluorescence imaging techniques, and fluorescence imaging instruments known to those of skill in the art. Examples of suitable fluorescence dyes that may be used (e.g., by conjugation to nucleotides, oligonucleotides, or proteins) include, but are not limited to, fluorescein, rhodamine, coumarin, cyanine, and derivatives thereof, including the cyanine derivatives Cyanine dye-3 (Cy3), Cyanine dye-5 (Cy5), Cyanine dye-7 (Cy7), etc. Examples of fluorescence imaging techniques that may be used include, but are not limited to, fluorescence microscopy imaging, fluorescence confocal imaging, two-photon fluorescence, and the like. Examples of fluorescence imaging instruments that may be used include, but are not limited to, fluorescence microscopes equipped with an image sensor or camera, confocal fluorescence microscopes, two-photon fluorescence microscopes, or custom instruments that comprise a suitable selection of light sources, lenses, mirrors, prisms, dichroic reflectors, apertures, and image sensors or cameras, etc. A non-limiting example of a fluorescence microscope equipped for acquiring images of the disclosed low-binding support surfaces and clonally-amplified colonies (colonies) of template nucleic acid sequences hybridized thereon is the Olympus IX83 inverted fluorescence microscope equipped with ) 20x, 0.75 NA, a 532 nm light source, a bandpass and dichroic mirror filter set optimized for 532 nm long-pass excitation and Cy3 fluorescence emission filter, a Semrock 532 nm dichroic reflector, and a camera (Andor sCMOS, Zyla 4.2) where the excitation light intensity is adjusted to avoid signal saturation. Often, the support surface may be immersed in a buffer (e.g., 25 mM ACES, pH 7.4 buffer) while the image is acquired.

**[0590]** In some instances, the performance of nucleic acid hybridization and/or amplification reactions using the disclosed reaction formulations and low non-specific binding supports may be assessed using fluorescence imaging techniques, where the contrast-to-noise ratio (CNR) of the images provides a key metric in assessing amplification

specificity and non-specific binding on the support. CNR is commonly defined as:  $CNR = (Signal - Background) / Noise$ . The background term is commonly taken to be the signal measured for the interstitial regions surrounding a particular feature (diffraction limited spot, DLS) in a specified region of interest (ROI). While signal-to-noise ratio (SNR) is often considered to be a benchmark of overall signal quality, it can be shown that improved CNR can provide a significant advantage over SNR as a benchmark for signal quality in applications that require rapid image capture (e.g., sequencing applications for which cycle times must be minimized), as shown in the example below. The surfaces of the instant disclosure are also provided in co-pending International Application Serial No. PCT/US2019/061556, which is hereby incorporated by reference in its entirety.

**[0591]** In most ensemble-based sequencing approaches, the background term is typically measured as the signal associated with ‘interstitial’ regions. In addition to “interstitial” background ( $B_{inter}$ ), “intrastitial” background ( $B_{intra}$ ) exists within the region occupied by an amplified DNA colony. The combination of these two background signals dictates the achievable CNR, and subsequently directly impacts the optical instrument requirements, architecture costs, reagent costs, run- times, cost/genome, and ultimately the accuracy and data quality for cyclic array-based sequencing applications. The  $B_{inter}$  background signal arises from a variety of sources; a few examples include auto-fluorescence from consumable flow cells, non-specific adsorption of detection molecules that yield spurious fluorescence signals that may obscure the signal from the ROI, the presence of non-specific DNA amplification products (e.g., those arising from primer dimers). In typical next generation sequencing (NGS) applications, this background signal in the current field-of-view (FOV) is averaged over time and subtracted. The signal arising from individual DNA colonies (i.e.,  $(S) - B_{inter}$  in the FOV) yields a discernable feature that can be classified. In some instances, the intrastitial background ( $B_{intra}$ ) can contribute a confounding fluorescence signal that is not specific to the target of interest, but is present in the same ROI thus making it far more difficult to average and subtract.

**[0592]** The implementation of nucleic acid amplification on the low-binding substrates of the present disclosure may decrease the  $B_{inter}$  background signal by reducing non-specific binding, may lead to improvements in specific nucleic acid amplification, and may lead to a decrease in non-specific amplification that can impact the background signal arising from both the interstitial and intrastitial regions. In some instances, the disclosed low-binding support surfaces, optionally used in combination with the disclosed hybridization buffer formulations, may lead to improvements in CNR by a factor of 2, 5, 10, 100, or 1000-fold

over those achieved using conventional supports and hybridization, amplification, and/or sequencing protocols. Although described here in the context of using fluorescence imaging as the read-out or detection mode, the same principles apply to the use of the disclosed low non-specific binding supports and nucleic acid hybridization and amplification formulations for other detection modes as well, including both optical and non-optical detection modes.

**[0593]** The disclosed low-binding supports, optionally used in combination with the disclosed hybridization and/or amplification protocols, yield solid-phase reactions that exhibit: (i) negligible non-specific binding of protein and other reaction components (thus minimizing substrate background), (ii) negligible non-specific nucleic acid amplification product, and (iii) provide tunable nucleic acid amplification reactions.

**[0594]** In some embodiments, fluorescence images of the disclosed low background surfaces when used in nucleic acid hybridization or amplification applications to create colonies of hybridized or clonally-amplified nucleic acid molecules (e.g., that have been directly or indirectly labeled with a fluorophore) exhibit contrast-to-noise ratios (CNRs) of at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, or greater than 250.

**[0595]** In some embodiments, a fluorescence image of the surface exhibits a contrast-to-noise ratio (CNR) of at least 20 when a sample nucleic acid molecule or complementary sequences thereof are labeled with a Cyanine dye-3 (Cy3) fluorophore, and when the fluorescence image is acquired using an inverted fluorescence microscope (e.g., Olympus IX83) with a  $20 \times 0.75$  NA objective, a 532 nm light source, a bandpass and dichroic mirror filter set optimized for 532 nm excitation and Cy3 fluorescence emission, and a camera (e.g., Andor sCMOS, Zyla 4.2) under non-signal saturating conditions while the surface is immersed in a buffer (e.g., 25 mM ACES, pH 7.4 buffer).

### **Hybridization Buffers**

**[0596]** The present disclosure provides compositions and methods for use of a hybridization buffer (e.g., high efficiency hybridization buffer) for distributing a plurality of covalently closed circular library molecules (600) onto a support having a plurality of immobilized fourth surface primers and optionally a plurality of third surface primers, so that individual covalently closed circular library molecules (600) hybridize to individual immobilized fourth and third surface primers thereby immobilizing the plurality of covalently closed circular library molecules (600).

**[0597]** The hybridization buffers (e.g., high efficiency hybridization buffers) comprise a first and second polar aprotic solvent, a pH buffer system and a crowding agent. The polar solvent as included in the hybridization composition described herein is a solvent or solvent system comprising one or more molecules characterized by the presence of a permanent dipole moment, i.e., a molecule having a spatially unequal distribution of charge density. A polar solvent may be characterized by a dielectric constant of 20, 25, 30, 35, 40, 45, 50, 55, 60 or by a value or a range of values having any of the aforementioned values. A polar solvent as described herein may comprise a polar aprotic solvent. A polar aprotic solvent as described herein may further contain no ionizable hydrogen in the molecule. In addition, polar solvents or polar aprotic solvents may be preferably substituted in the context of the presently disclosed compositions with a strong polarizing functional groups such as nitrile, carbonyl, thiol, lactone, sulfone, sulfite, and carbonate groups so that the underlying solvent molecules have a dipole moment. Polar solvents and polar aprotic solvents can be present in both aliphatic and aromatic or cyclic form. In some embodiments, the polar solvent is acetonitrile.

**[0598]** The polar or polar aprotic solvent described herein can have a dielectric constant that is the same as or close to acetonitrile. The dielectric constant of the polar or polar aprotic solvent can be in the range of about 20-60, about 25-55, about 25-50, about 25-45, about 25-40, about 30-50, about 30-45, or about 30-40. The dielectric constant of the polar or polar aprotic solvent can be greater than 20, 25, 30, 35, or 40. The dielectric constant of the polar or polar aprotic solvent can be lower than 30, 40, 45, 50, 55, or 60. The dielectric constant of the polar or polar aprotic solvent can be about 35, 36, 37, 38, or 39.

**[0599]** The polar or polar aprotic solvent described herein can have a polarity index that is the same as or close to acetonitrile. The polarity index of the polar or polar aprotic solvent can be in the range of about 2-9, 2-8, 2-7, 2-6, 3-9, 3-8, 3-7, 3-6, 4-9, 4-8, 4-7, or 4-6. The polarity index of the polar or polar aprotic solvent can be greater than about 2, 3, 4, 4.5, 5, 5.5, or 6. The polarity index of the polar or polar aprotic solvent can be lower than about 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, or 10. The polarity index of the polar or polar aprotic solvent can be about 5.5, 5.6, 5.7, or 5.8.

**[0600]** Some examples of the polar or polar aprotic solvent include but are not limited to acetonitrile, dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetanilide, N-acetyl pyrrolidone, 4-amino pyridine, benzamide, benzimidazole, 1,2,3-benzotriazole, butadienedioxide, 2,3-butylene carbonate,  $\gamma$ -butyrolactone, caprolactone (epsilon), chloro maleic anhydride, 2-chlorocyclohexanone, chloroethylene carbonate, chloronitromethane,

citraconic anhydride, crotonlactone, 5-cyano-2-thiouracil, cyclopropyl nitrile, dimethyl sulfate, dimethyl sulfone, 1,3-dimethyl-5-tetrazole, 1,5-dimethyl tetrazole, 1,2-dinitrobenzene, 2,4-dinitrotoluene, dipheynyl sulfone, 1,2-dinitrobenzene, 2,4-dinitrotoluene, dipheynyl sulfone, epsilon-caprolactam, ethanesulfonylchloride, ethyl ethyl phosphinate, N-ethyl tetrazole, ethylene carbonate, ethylene trithiocarbonate, ethylene glycol sulfate, ethylene glycol sulfite, furfural, 2-furonitrile, 2-imidazole, isatin, isoxazole, malononitrile, 4-methoxy benzonitrile, 1-methoxy-2-nitrobenzene, methyl alpha bromo tetronate, 1 -methyl imidazole, N-methyl imidazole, 3 -methyl isoxazole, N-methyl morpholine-N-oxide, methyl phenyl sulfone, N-methyl pyrrolidinone, methyl sulfolane, methyl-4-toluenesulfonate, 3-nitroaniline, nitrobenzimidazole, 2-nitrofuran, 1-nitroso-2-pyrrolidinone, 2-nitrothiophene, 2-oxazolidinone, 9,10-phenanthrenequinone, N-phenyl sydnone, phthalic anhydride, picolinonitrile (2-cyanopyridine), 1,3-propane sultone,  $\beta$ -propiolactone, propylene carbonate, 4H-pyran-4-thione, 4H-pyran-4-one ( $\gamma$ -pyrone), pyridazine, 2-pyrrolidone, saccharin, succinonitrile, sulfanilamide, sulfolane, 2,2,6,6-tetrachlorocyclohexanone, tetrahydrothiapyran oxide, tetramethylene sulfone (sulfolane), thiazole, 2-thiouracil, 3,3,3-trichloro propene, 1,1,2-trichloro propene, 1,2,3-trichloro propene, trimethylene sulfide-dioxide, and trimethylene sulfite.

**[0601]** The amount of the polar solvent or polar aprotic solvent is present in an amount effective to denature a double stranded nucleic acid. In some embodiments, the amount of the polar or polar aprotic solvent is greater than about 10% by volume based on the total volume of the formulation. The amount of the polar or polar aprotic solvent is about or more than about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 70%, 80%, 90%, or higher, by volume based on the total volume of the formulation. The amount of the polar or polar aprotic solvent is lower than about 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 70%, 80%, 90%, or higher, by volume based on the total volume of the formulation. In some embodiments, the amount of the polar or polar aprotic solvent is in the range of about 10% to 90% by volume based on the total volume of the formulation. In some embodiments, the amount of the polar or polar aprotic solvent is in the range of about 25% to 75% by volume based on the total volume of the formulation. In some embodiments, the amount of the polar or polar aprotic solvent is in the range of about 10% to 95%, 10% to 85%, 20% to 90%, 20% to 80%, 20% to 75%, or 30% to 60% by volume based on the total volume of the formulation.

**[0602]** In some embodiments, the disclosed hybridization buffer formulations may include the addition of an organic solvent. Examples of suitable solvents include, but are not limited

to, acetonitrile, ethanol, DMF, and methanol, or any combination thereof at varying percentages (typically > 5%). In some embodiments, the percentage of organic solvent (by volume) included in the hybridization buffer may range from about 1% to about 20%. In some embodiments, the percentage by volume of organic solvent may be at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, or at least 20%. In some embodiments, the percentage by volume of organic solvent may be at most 20%, at most 15%, at most 10%, at most 9%, at most 8%, at most 7%, at most 6%, at most 5%, at most 4%, at most 3%, at most 2%, or at most 1%. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, the percentage by volume of organic solvent may range from about 4% to about 15%. Those of skill in the art will recognize that the percentage by volume of organic solvent may have any value within this range, e.g., about 7.5%.

**[0603]** Improvements in hybridization rate: In some embodiments, the use of optimized buffer formulations disclosed herein (optionally, used in combination with low non-specific binding surface) yield relative hybridization rates that range from about 2x to about 20x faster than that for a conventional hybridization protocol. In some embodiments, the relative hybridization rate may be at least 2x, at least 3x, at least 4x, at least 5x, at least 6x, at least 7x, at least 8x, at least 9x, at least 10x, at least 12x, at least 14x, at least 16x, at least 18x, or at least 20x that for a conventional hybridization protocol.

**[0604]** Improvements in hybridization efficiency (or yield) is a measure of the percentage of total available tethered adaptor sequences on a solid surface, primer sequences, or oligonucleotide sequences in general that are hybridized to complementary sequences. In some embodiments, the use of optimized buffer formulations disclosed herein (optionally, used in combination with low non-specific binding surface) yield improved hybridization efficiency compared to that for a conventional hybridization protocol. In some embodiments, the hybridization efficiency that may be achieved is better than 80%, 85%, 90%, 95%, 98%, or 99% in any of the hybridization reaction times specified above.

**[0605]** Improvements in hybridization specificity is a measure of the ability of tethered adaptor sequences, primer sequences, or oligonucleotide sequences in general to correctly hybridize only to completely complementary sequences. In some embodiments, the use of the optimized buffer formulations disclosed herein (optionally, used in combination with low non-specific binding surface) yield improved hybridization specificity compared to that for a conventional hybridization protocol. In some embodiments, the hybridization specificity that

may be achieved is better than 1 base mismatch in 10 hybridization events, 1 base mismatch in 100 hybridization events, 1 base mismatch in 1,000 hybridization events, or 1 base mismatch in 10,000 hybridization events.

**[0606]** The term “crowding agent” and related terms refers to a compound that alters the properties of other molecules in a solution. Crowding agents typically have high molecular weight and/or bulky structures. Crowding agents in solution can increase the concentration of other molecules in the solution. Crowding agents can reduce the volume of solvent that is available for other molecules in the solution which can create a molecular crowding environment. Crowding agents in a solution can generate a crowded environment for molecules in the solution. Crowding agents can alter the rates or equilibrium constants of a reaction. Examples of crowding agents include polyethylene glycol (e.g., PEG), ficoll, dextran, glycogen, polyvinyl alcohol, triblock polymers (e.g., Pluronics), polystyrene, polyvinylpyrrolidone (PVP), hydroxypropyl methyl cellulose (HPMC), hydroxyethyl methyl cellulose (HEMC), hydroxybutyl methyl cellulose, hydroxypropyl cellulose, methycellulose, and hydroxyl methyl cellulose. In some embodiments, the crowding agent comprises linear or branched PEG. In some embodiments, the crowding agent comprise PEG 400, PEG 1500, PEG 2000, PEG 3400, PEG 3350, PEG 4000, PEG 6000 or PEG 8000. In some embodiments, a solution can include at least one crowding agent at about 1%, 2%, 3%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, or higher percent based on volume of the solution. In some embodiments, the solution can be used for nucleic acid amplification including rolling circle amplification and/or multiple displacement amplification reactions.

**[0607]** A suitable amount of a crowding agent in the composition allows for, enhances, or facilitates molecular crowding. The amount of the crowding agent is about or more than about 1%, 2%, 3%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, or higher, by volume based on the total volume of the formulation. In some cases, the amount of the molecular crowding agent is greater than 5% by volume based on the total volume of the formulation. The amount of the crowding agent is lower than about 3%, 5%, 10%, 12.5%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 70%, 80%, 90%, or higher, by volume based on the total volume of the formulation. In some cases, the amount of the molecular crowding agent can be less than 30% by volume based on the total volume of the formulation. In some embodiments, the amount of the polar or polar aprotic solvent is in the range of about 25% to 75% by volume based on the total volume of the formulation. In some embodiments, the amount of the polar or polar aprotic solvent is in the range of about 1% to 40%, 1% to 35%, 2% to 50%, 2% to 40%, 2% to 35%, 2% to 30%, 2% to 25%, 2% to 20%,

2% to 10%, 5% to 50%, 5% to 40%, 5% to 35%, 5% to 30%, 5% to 25%, 5% to 20%, by volume based on the total volume of the formulation. In some cases, the amount of the molecular crowding agent can be in the range of about 5% to about 20% by volume based on the total volume of the formulation. In some embodiments, the amount of the crowding agent is in the range of about 1% to 30% by volume based on the total volume of the formulation.

**[0608]** In some embodiments, the disclosed hybridization buffer formulations may include the addition of a molecular crowding or volume exclusion agent. Molecular crowding or volume exclusion agents are typically macromolecules (e.g., proteins) which, when added to a solution in high concentrations, may alter the properties of other molecules in solution by reducing the volume of solvent available to the other molecules. In some embodiments, the percentage by volume of molecular crowding or volume exclusion agent included in the hybridization buffer formulation may range from about 1% to about 50%. In some embodiments, the percentage by volume of molecular crowding or volume exclusion agent may be at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, or at least 50%. In some embodiments, the percentage by volume of molecular crowding or volume exclusion agent may be at most 50%, at most 45%, at most 40%, at most 35%, at most 30%, at most 25%, at most 20%, at most 15%, at most 10%, at most 5%, or at most 1%. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, the percentage by volume of molecular crowding or volume exclusion agent may range from about 5% to about 35%. Those of skill in the art will recognize that the percentage by volume of molecular crowding or volume exclusion agent may have any value within this range, e.g., about 12.5%.

**[0609]** The hybridization buffer described herein includes a pH buffer system that maintains the pH of the compositions in a range suitable for hybridization process. The pH buffer system can include one or more buffering agents selected from the group consisting of Tris, HEPES, TAPS, Tricine, Bicine, Bis-Tris, NaOH, KOH, TES, EPPS, MES, and MOPS. The pH buffer system can further include a solvent. A preferred pH buffer system includes MOPS, MES, TAPS, phosphate buffer combined with methanol, acetonitrile, ethanol, isopropanol, butanol, t-butyl alcohol, DMF, DMSO, or any combination therein.

**[0610]** The hybridization buffer includes an amount of the pH buffer system that is effective to maintain the pH of the formulation to be in a range suitable for the hybridization. In some embodiments, the pH may be at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10. In some embodiments, the pH may be at most 10, at most 9,

at most 8, at most 7, at most 6, at most 5, at most 4, or at most 3. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, the pH of the hybridization buffer may range from about 4 to about 8. Those of skill in the art will recognize that the pH of the hybridization buffer may have any value within this range, e.g., about pH 7.8. In some cases, the pH range is about 3 to about 10. In some embodiments, the disclosed hybridization buffer formulations may include adjustment of pH over the range of about pH 3 to pH 10, with a preferred buffer range of 5-9.

**[0611]** The hybridization buffer described herein includes an additive (e.g., polar aprotic solvent) for controlling melting temperature of nucleic acid can vary depending on other agents used in the compositions. The amount of the additive for controlling melting temperature of the nucleic acid is about or more than about 1%, 2%, 3%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, or higher, by volume based on the total volume of the formulation. In some cases, the amount of the additive for controlling melting temperature of the nucleic acid is greater than about 2% by volume based on the total volume of the formulation. In some cases, the amount of the additive for controlling melting temperature of the nucleic acid is greater than 5% by volume based on the total volume of the formulation. In some cases, the amount of the additive for controlling melting temperature of the nucleic acid is lower than about 3%, 5%, 10%, 12.5%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 70%, 80%, 90%, or higher, by volume based on the total volume of the formulation. In some embodiments, the amount of the additive for controlling melting temperature of the nucleic acid is in the range of about 1% to 40%, 1% to 35%, 2% to 50%, 2% to 40%, 2% to 35%, 2% to 30%, 2% to 25%, 2% to 20%, 2% to 10%, 5% to 50%, 5% to 40%, 5% to 35%, 5% to 30%, 5% to 25%, 5% to 20%, by volume based on the total volume of the formulation. In some embodiments, the amount of the additive for controlling melting temperature of the nucleic acid is in the range of about 2% to 20% by volume based on the total volume of the formulation. In some cases, the amount of the additive for controlling melting temperature of the nucleic acid is in the range of about 5% to 10% by volume based on the total volume of the formulation.

**[0612]** In some embodiments, the disclosed hybridization buffer formulations may include the addition of an additive that alters nucleic acid duplex melting temperature. Examples of suitable additives that may be used to alter nucleic acid melting temperature include, but are not limited to, Formamide. In some embodiments, the percentage by volume of a melting temperature additive included in the hybridization buffer formulation may range from about 1% to about 50%. In some embodiments, the percentage by volume of a melting temperature

additive may be at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, or at least 50%. In some embodiments, the percentage by volume of a melting temperature additive may be at most 50%, at most 45%, at most 40%, at most 35%, at most 30%, at most 25%, at most 20%, at most 15%, at most 10%, at most 5%, or at most 1%. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, the percentage by volume of a melting temperature additive may range from about 10% to about 25%. Those of skill in the art will recognize that the percentage by volume of a melting temperature additive may have any value within this range, e.g., about 22.5%.

**[0613]** In some embodiments, the hybridization buffer described herein includes an additive that impacts DNA hydration: In some embodiments, the disclosed hybridization buffer formulations may include the addition of an additive that impacts nucleic acid hydration. Examples include, but are not limited to, betaine, urea, glycine betaine, or any combination thereof. In some embodiments, the percentage by volume of a hydration additive included in the hybridization buffer formulation may range from about 1% to about 50%. In some embodiments, the percentage by volume of a hydration additive may be at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, or at least 50%. In some embodiments, the percentage by volume of a hydration additive may be at most 50%, at most 45%, at most 40%, at most 35%, at most 30%, at most 25%, at most 20%, at most 15%, at most 10%, at most 5%, or at most 1%. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, the percentage by volume of a hydration additive may range from about 1% to about 30%. Those of skill in the art will recognize that the percentage by volume of a melting temperature additive may have any value within this range, e.g., about 6.5%.

### **Sequencing Polymerases**

**[0614]** The present disclosure provides methods for sequencing nucleic acid molecules, wherein any of the sequencing methods described herein employ at least one type of sequencing polymerase and a plurality of nucleotides, or employ at least one type of sequencing polymerase and a plurality of nucleotides and a plurality of multivalent molecules. In some embodiments, the sequencing polymerase(s) is/are capable of

incorporating a complementary nucleotide opposite a nucleotide in a concatemer template molecule. In some embodiments, the sequencing polymerase(s) is/are capable of binding a complementary nucleotide unit of a multivalent molecule opposite a nucleotide in a concatemer template molecule. In some embodiments, the plurality of sequencing polymerases comprise recombinant mutant polymerases.

**[0615]** Examples of suitable polymerases for use in sequencing with nucleotides and/or multivalent molecules include but are not limited to: Klenow DNA polymerase; *Thermus aquaticus* DNA polymerase I (Taq polymerase); KlenTaq polymerase; *Candidatus altiarthaeales* archaeon; *Candidatus Hadarchaeum Yellowstoneense*; *Hadesarchaea* archaeon; *Euryarchaeota* archaeon; *Thermoplasmata* archaeon; *Thermococcus* polymerases such as *Thermococcus litoralis*, bacteriophage T7 DNA polymerase; human alpha, delta and epsilon DNA polymerases; bacteriophage polymerases such as T4, RB69 and phi29 bacteriophage DNA polymerases; *Pyrococcus furiosus* DNA polymerase (Pfu polymerase); *Bacillus subtilis* DNA polymerase III; *E. coli* DNA polymerase III alpha and epsilon; 9 degree N polymerase; reverse transcriptases such as HIV type M or O reverse transcriptases; avian myeloblastosis virus reverse transcriptase; Moloney Murine Leukemia Virus (MMLV) reverse transcriptase; or telomerase. Further non-limiting examples of DNA polymerases include those from various Archaea genera, such as, *Aeropyrum*, *Archaeoglobus*, *Desulfurococcus*, *Pyrobaculum*, *Pyrococcus*, *Pyrolobus*, *Pyrodictium*, *Staphylothermus*, *Stetteria*, *Sulfolobus*, *Thermococcus*, and *Vulcanisaeta* and the like or variants thereof, including such polymerases as are known in the art such as 9 degrees N, VENT, DEEP VENT, THERMINATOR, Pfu, KOD, Pfx, Tgo and RB69 polymerases.

### **Nucleotides**

**[0616]** The present disclosure provides methods for sequencing nucleic acid molecules using nucleotides, wherein at least one nucleotide in the plurality of nucleotides comprise a base, sugar and at least one phosphate group. In some embodiments, at least one nucleotide in the plurality comprises an aromatic base, a five carbon sugar (e.g., ribose or deoxyribose), and one or more phosphate groups (e.g., 1-10 phosphate groups). The plurality of nucleotides can comprise at least one type of nucleotide selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP. The plurality of nucleotides can comprise a mixture of any combination of two or more types of nucleotides selected from a group consisting of dATP, dGTP, dCTP, dTTP and/or dUTP. In some embodiments, at least one nucleotide in the

plurality is not a nucleotide analog. In some embodiments, at least one nucleotide in the plurality comprises a nucleotide analog.

**[0617]** In some embodiments, in any of the methods for sequencing nucleic acid molecules described herein, at least one nucleotide in the plurality of nucleotides comprise a chain of one, two or three phosphorus atoms where the chain is typically attached to the 5' carbon of the sugar moiety via an ester or phosphoramidate linkage. In some embodiments, at least one nucleotide in the plurality is an analog having a phosphorus chain in which the phosphorus atoms are linked together with intervening O, S, NH, methylene or ethylene. In some embodiments, the phosphorus atoms in the chain include substituted side groups including O, S or BH<sub>3</sub>. In some embodiments, the chain includes phosphate groups substituted with analogs including phosphoramidate, phosphorothioate, phosphordithioate, and O-methylphosphoroamidite groups.

**[0618]** In some embodiments, in any of the methods for sequencing nucleic acid molecules described herein, at least one nucleotide in the plurality of nucleotides comprises a terminator nucleotide analog having a chain terminating moiety (e.g., blocking moiety) at the sugar 2' position, at the sugar 3' position, or at the sugar 2' and 3' position. In some embodiments, the chain terminating moiety can inhibit polymerase-catalyzed incorporation of a subsequent nucleotide unit or free nucleotide in a nascent strand during a primer extension reaction. In some embodiments, the chain terminating moiety is attached to the 3' sugar hydroxyl position where the sugar comprises a ribose or deoxyribose sugar moiety. In some embodiments, the chain terminating moiety is removable/cleavable from the 3' sugar hydroxyl position to generate a nucleotide having a 3'OH sugar group which is extendible with a subsequent nucleotide in a polymerase-catalyzed nucleotide incorporation reaction. In some embodiments, 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. In some embodiments, the chain terminating moiety is cleavable/removable from the nucleotide, for example by reacting the chain terminating moiety with a chemical agent, pH change, light or heat. In some embodiments, the chain terminating moieties alkyl, alkenyl, alkynyl and allyl are cleavable with tetrakis(triphenylphosphine)palladium(0) (Pd(PPh<sub>3</sub>)<sub>4</sub>) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). In some embodiments, the chain terminating moieties aryl and benzyl are cleavable with H<sub>2</sub> Pd/C. In some embodiments, the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable

with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). In some embodiments, the chain terminating moiety carbonate is cleavable with potassium carbonate ( $K_2CO_3$ ) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). In some embodiments, the chain terminating moieties urea and silyl are cleavable with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

**[0619]** In some embodiments, in any of the methods for sequencing nucleic acid molecules described herein, at least one nucleotide in the plurality of nucleotides comprises a terminator nucleotide analog having a chain terminating moiety (e.g., blocking moiety) at the sugar 2' position, at the sugar 3' position, or at the sugar 2' and 3' position. In some embodiments, the chain terminating moiety comprises an azide, azido or azidomethyl group. In some embodiments, the chain terminating moiety comprises a 3'-O-azido or 3'-O-azidomethyl group. In some embodiments, the chain terminating moieties azide, azido and azidomethyl group are cleavable/removable with a phosphine compound. In some embodiments, the phosphine compound comprises a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound comprises Tris(2-carboxyethyl)phosphine (TCEP) or bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxypropyl)phosphine (THPP). In some embodiments, the cleaving agent comprises 4-dimethylaminopyridine (4-DMAP).

**[0620]** In some embodiments, in any of the methods for sequencing nucleic acid molecules described herein, the nucleotide comprises a chain terminating moiety which is selected from a group consisting of 3'-deoxy nucleotides, 2',3'-dideoxynucleotides, 3'-methyl, 3'-azido, 3'-azidomethyl, 3'-O-azidoalkyl, 3'-O-ethynyl, 3'-O-aminoalkyl, 3'-O-fluoroalkyl, 3'-fluoromethyl, 3'-difluoromethyl, 3'-trifluoromethyl, 3'-sulfonyl, 3'-malonyl, 3'-amino, 3'-O-amino, 3'-sulfhydryl, 3'-aminomethyl, 3'-ethyl, 3'-butyl, 3'-*tert* butyl, 3'-Fluorenylmethyloxycarbonyl, 3'-*tert*-Butyloxycarbonyl, 3'-O-alkyl hydroxylamino group, 3'-phosphorothioate, and 3-O-benzyl, or derivatives thereof.

**[0621]** In some embodiments, in any of the methods for sequencing nucleic acid molecules described herein, the plurality of nucleotides comprises a plurality of nucleotides labeled with detectable reporter moiety. The detectable reporter moiety comprises a fluorophore. In some embodiments, the fluorophore is attached to the nucleotide base. In some embodiments, the fluorophore is attached to the nucleotide base with a linker which is cleavable/removable from the base. In some embodiments, at least one of the nucleotides in the plurality is not labeled with a detectable reporter moiety. In some embodiments, a particular detectable

reporter moiety (e.g., fluorophore) that is attached to the nucleotide can correspond to the nucleotide base (e.g., dATP, dGTP, dCTP, dTTP or dUTP) to permit detection and identification of the nucleotide base.

**[0622]** In some embodiments, in any of the methods for sequencing nucleic acid molecules described herein, the cleavable linker on the nucleotide base comprises a cleavable moiety comprising 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. In some embodiments, the cleavable linker on the base is cleavable/removable from the base by reacting the cleavable moiety with a chemical agent, pH change, light or heat. In some embodiments, the cleavable moieties alkyl, alkenyl, alkynyl and allyl are cleavable with tetrakis(triphenylphosphine)palladium(0) ( $\text{Pd(PPh}_3\text{)}_4$ ) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). In some embodiments, the cleavable moieties aryl and benzyl are cleavable with  $\text{H}_2$  Pd/C. In some embodiments, the cleavable moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). In some embodiments, the cleavable moiety carbonate is cleavable with potassium carbonate ( $\text{K}_2\text{CO}_3$ ) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). In some embodiments, the cleavable moieties urea and silyl are cleavable with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

**[0623]** In some embodiments, in any of the methods for sequencing nucleic acid molecules described herein, the cleavable linker on the nucleotide base comprises cleavable moiety including an azide, azido or azidomethyl group. In some embodiments, the cleavable moieties azide, azido and azidomethyl group are cleavable/removable with a phosphine compound. In some embodiments, the phosphine compound comprises a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound comprises Tris(2-carboxyethyl)phosphine (TCEP) or bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxypropyl)phosphine (THPP). In some embodiments, the cleaving agent comprises 4-dimethylaminopyridine (4-DMAP).

**[0624]** In some embodiments, in any of the methods for sequencing nucleic acid molecules described herein, the chain terminating moiety (e.g., at the sugar 2' and/or sugar 3' position) and the cleavable linker on the nucleotide base have the same or different cleavable moieties. In some embodiments, the chain terminating moiety (e.g., at the sugar 2' and/or sugar 3' position) and the detectable reporter moiety linked to the base are chemically

cleavable/removable with the same chemical agent. In some embodiments, the chain terminating moiety (e.g., at the sugar 2' and/or sugar 3' position) and the detectable reporter moiety linked to the base are chemically cleavable/removable with different chemical agents.

### **Multivalent Molecules**

**[0625]** The present disclosure provides methods for sequencing nucleic acid molecules which employ multivalent molecules. In some embodiments, the multivalent molecule comprises a plurality of nucleotide arms attached to a core and having any configuration including a starburst, helter skelter, or bottle brush configuration (e.g., FIGS. 12-15). The multivalent molecule comprises: (1) a core; and (2) 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, wherein the spacer is attached to the linker, wherein the linker is attached to the nucleotide unit. In some embodiments, the nucleotide unit comprises a base, sugar and at least one phosphate group, and the linker is attached to the nucleotide unit through the base. In some embodiments, the linker comprises an aliphatic chain or an oligo ethylene glycol chain where both linker chains having 2-6 subunits. In some embodiments, the linker also includes an aromatic moiety. An exemplary nucleotide arm is shown in FIG. 16. Exemplary multivalent molecules are shown in FIGS. 12-15. An exemplary spacer is shown in FIG. 17 (top) and exemplary linkers are shown in FIG. 17 (bottom) and FIG. 18. Exemplary nucleotides attached to a linker are shown in FIGS. 19-21. An exemplary biotinylated nucleotide arm is shown in FIG. 22.

**[0626]** In some embodiments, a multivalent molecule comprises a core attached to multiple nucleotide arms, and wherein the multiple nucleotide arms have the same type of nucleotide unit which is selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

**[0627]** In some embodiments, a multivalent molecule comprises a core attached to multiple nucleotide arms, where each arm includes a nucleotide unit. The nucleotide unit comprises an aromatic base, a five carbon sugar (e.g., ribose or deoxyribose), and one or more phosphate groups (e.g., 1-10 phosphate groups). The plurality of multivalent molecules can comprise one type multivalent molecule having one type of nucleotide unit selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP. The plurality of multivalent molecules can comprise at a mixture of any combination of two or more types of multivalent molecules, where individual multivalent molecules in the mixture comprise nucleotide units selected from a group consisting of dATP, dGTP, dCTP, dTTP and/or dUTP.

**[0628]** In some embodiments, the nucleotide unit comprises a chain of one, two or three phosphorus atoms where the chain is typically attached to the 5' carbon of the sugar moiety via an ester or phosphoramidate linkage. In some embodiments, at least one nucleotide unit is a nucleotide analog having a phosphorus chain in which the phosphorus atoms are linked together with intervening O, S, NH, methylene or ethylene. In some embodiments, the phosphorus atoms in the chain include substituted side groups including O, S or BH<sub>3</sub>. In some embodiments, the chain includes phosphate groups substituted with analogs including phosphoramidate, phosphorothioate, phosphordithioate, and O-methylphosphoroamidite groups.

**[0629]** In some embodiments, the multivalent molecule comprises a core attached to multiple nucleotide arms, and wherein individual nucleotide arms comprise a nucleotide unit which is a nucleotide analog having a chain terminating moiety (e.g., blocking moiety) at the sugar 2' position, at the sugar 3' position, or at the sugar 2' and 3' position. In some embodiments, the nucleotide unit comprises a chain terminating moiety (e.g., blocking moiety) at the sugar 2' position, at the sugar 3' position, or at the sugar 2' and 3' position. In some embodiments, the chain terminating moiety can inhibit polymerase-catalyzed incorporation of a subsequent nucleotide unit or free nucleotide in a nascent strand during a primer extension reaction. In some embodiments, the chain terminating moiety is attached to the 3' sugar hydroxyl position where the sugar comprises a ribose or deoxyribose sugar moiety. In some embodiments, the chain terminating moiety is removable/cleavable from the 3' sugar hydroxyl position to generate a nucleotide having a 3'OH sugar group which is extendible with a subsequent nucleotide in a polymerase-catalyzed nucleotide incorporation reaction. In some embodiments, 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. In some embodiments, the chain terminating moiety is cleavable/removable from the nucleotide unit, for example by reacting the chain terminating moiety with a chemical agent, pH change, light or heat. In some embodiments, the chain terminating moieties alkyl, alkenyl, alkynyl and allyl are cleavable with tetrakis(triphenylphosphine)palladium(0) (Pd(PPh<sub>3</sub>)<sub>4</sub>) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). In some embodiments, the chain terminating moieties aryl and benzyl are cleavable with H<sub>2</sub> Pd/C. In some embodiments, the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable with phosphine or with a thiol group including beta-mercaptoethanol or

dithiothritol (DTT). In some embodiments, the chain terminating moiety carbonate is cleavable with potassium carbonate ( $K_2CO_3$ ) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). In some embodiments, the chain terminating moieties urea and silyl are cleavable with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

**[0630]** In some embodiments, the nucleotide unit comprises a chain terminating moiety (e.g., blocking moiety) at the sugar 2' position, at the sugar 3' position, or at the sugar 2' and 3' position. In some embodiments, the chain terminating moiety comprises an azide, azido or azidomethyl group. In some embodiments, the chain terminating moiety comprises a 3'-O-azido or 3'-O-azidomethyl group. In some embodiments, the chain terminating moieties azide, azido and azidomethyl group are cleavable/removable with a phosphine compound. In some embodiments, the phosphine compound comprises a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound comprises Tris(2-carboxyethyl)phosphine (TCEP) or bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxypropyl)phosphine (THPP). In some embodiments, the cleaving agent comprises 4-dimethylaminopyridine (4-DMAP).

**[0631]** In some embodiments, the nucleotide unit comprising a chain terminating moiety which is selected from a group consisting of 3'-deoxy nucleotides, 2',3'-dideoxynucleotides, 3'-methyl, 3'-azido, 3'-azidomethyl, 3'-O-azidoalkyl, 3'-O-ethynyl, 3'-O-aminoalkyl, 3'-O-fluoroalkyl, 3'-fluoromethyl, 3'-difluoromethyl, 3'-trifluoromethyl, 3'-sulfonyl, 3'-malonyl, 3'-amino, 3'-O-amino, 3'-sulfhydryl, 3'-aminomethyl, 3'-ethyl, 3'-butyl, 3'-*tert* butyl, 3'-Fluorenylmethyloxycarbonyl, 3'-*tert*-Butyloxycarbonyl, 3'-O-alkyl hydroxylamino group, 3'-phosphorothioate, and 3-O-benzyl, or derivatives thereof.

**[0632]** In some embodiments, the multivalent molecule comprises a core attached to multiple nucleotide arms, wherein the nucleotide arms comprise a spacer, linker and nucleotide unit, and wherein the core, linker and/or nucleotide unit is labeled with detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore. In some embodiments, a particular detectable reporter moiety (e.g., fluorophore) that is attached to the multivalent molecule can correspond to the base (e.g., dATP, dGTP, dCTP, dTTP or dUTP) of the nucleotide unit to permit detection and identification of the nucleotide base.

**[0633]** In some embodiments, at least one nucleotide arm of a multivalent molecule has a nucleotide unit that is attached to a detectable reporter moiety. In some embodiments, the detectable reporter moiety is attached to the nucleotide base. In some embodiments, the

detectable reporter moiety comprises a fluorophore. In some embodiments, a particular detectable reporter moiety (e.g., fluorophore) that is attached to the multivalent molecule can correspond to the base (e.g., dATP, dGTP, dCTP, dTTP or dUTP) of the nucleotide unit to permit detection and identification of the nucleotide base.

**[0634]** In some embodiments, the core of a multivalent molecule comprises an avidin-like or streptavidin-like moiety and the core attachment moiety comprises biotin. In some embodiments, the core comprises an streptavidin-type or avidin-type moiety which includes an avidin protein, as well as any derivatives, analogs and other non-native forms of avidin that can bind to at least one biotin moiety. Other forms of avidin moieties include native and recombinant avidin and streptavidin as well as derivatized molecules, e.g. non-glycosylated avidin and truncated streptavidins. For example, avidin moiety includes de-glycosylated forms of avidin, bacterial streptavidin produced by *Streptomyces* (e.g., *Streptomyces avidinii*), as well as derivatized forms, for example, N-acyl avidins, e.g., N-acetyl, N-phthalyl and N-succinyl avidin, and the commercially-available products EXTRAVIDIN, CAPTAVIDIN, NEUTRAVIDIN and NEUTRALITE AVIDIN.

**[0635]** In some embodiments, any of the methods for sequencing nucleic acid molecules described herein can include forming a binding complex, where the binding complex comprises (i) a polymerase, a nucleic acid concatemer molecule duplexed with a primer, and a nucleotide, or the binding complex comprises (ii) a polymerase, a nucleic acid concatemer molecule duplexed with a primer, and a nucleotide unit of a multivalent molecule. In some embodiments, the binding complex has a persistence time of greater than about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1 second. The binding complex has a persistence time of greater than about 0.1-0.25 seconds, or about 0.25-0.5 seconds, or about 0.5-0.75 seconds, or about 0.75-1 second, or about 1-2 seconds, or about 2-3 seconds, or about 3-4 second, or about 4-5 seconds, and/or wherein the method is or may be carried out at a temperature of at or above 15 °C, at or above 20 °C, at or above 25 °C, at or above 35 °C, at or above 37 °C, at or above 42 °C at or above 55 °C at or above 60 °C, or at or above 72 °C, or at or above 80 °C, or within a range defined by any of the foregoing. The binding complex (e.g., ternary complex) remains stable until subjected to a condition that causes dissociation of interactions between any of the polymerase, template molecule, primer and/or the nucleotide unit or the nucleotide. For example, a dissociating condition comprises contacting the binding complex with any one or any combination of a detergent, EDTA and/or water. In some embodiments, the present disclosure provides said method wherein the binding complex is deposited on,

attached to, or hybridized to, a surface showing a contrast to noise ratio in the detecting step of greater than 20. In some embodiments, the present disclosure provides said method wherein the contacting is performed under a condition that stabilizes the binding complex when the nucleotide or nucleotide unit is complementary to a next base of the template nucleic acid, and destabilizes the binding complex when the nucleotide or nucleotide unit is not complementary to the next base of the template nucleic acid.

## EXAMPLES

**[0636]** The following examples are meant to be illustrative and can be used to further understand embodiments of the present disclosure and should not be construed as limiting the scope of the present teachings in any way.

### **[0637] EXAMPLE 1: Linear Nucleic Acid Libraries**

**[0638]** Commercially-available kits were used to prepare linear nucleic acid libraries having a sequence of interest (insert) appended to at least one universal sequencing primer, and universal binding sequences P5 (e.g., first surface primer) and P7 (e.g., second surface primer) at their terminal ends. The commercially-available kits are intended for preparing library molecules for a next gen sequencing platform such as Illumina. Input nucleic acids containing the sequences of interest included DNA and RNA from various organisms. The input nucleic acids were sheared using Covaris to achieve insert sizes of about 200, 250, 350 and 400 bp. The amount of input fragmented nucleic acids used to prepare the linear library molecules included 50 ng, 100 ng, 500 ng and 1 ug.

**[0639]** The input nucleic acids were obtained from *Haemophilus influenzae* (38% GC); *E. coli* (51% GC); *Rhodopseudomonas palustris* (65% GC); PhiX; Human; Human exome; and Human mRNA. A metagenomic DNA sample was also used to prepare linear library molecules. The metagenomic sample contained approximately 10% DNA of each of the following: *Bacillus cereus* (ATCC 10987); *Bifidobacterium adolescentis* (ATCC 15703); *Clostridium beijerinckii* (ATCC 35702); *Deinococcus radiodurans* (ATCC BAA-816); *Enterococcus faecalis* (ATCC 47077); *Escherichia coli* (ATCC 700926); *Lactobacillus gasseri* (ATCC 33323); *Rhodobacter sphaeroides* (ATCC 17029); *Staphylococcus epidermidis* (ATCC 12228); and *Streptococcus mutans* (ATCC 700610).

### **[0640] EXAMPLE 2: Preparing Library-Splint Complexes**

**[0641]** Linear library preps (e.g., 0.25, 0.5 or 1 pmol) were annealed to the double-stranded splint adaptors (200) in an annealing buffer containing 100 mM potassium acetate and 30

mM HEPES (pH 7.5), in a thermal cycler apparatus. The annealing program included: 5 minutes at 95 degrees C, 5 minutes at 37 degrees C, and hold at 37 degrees C.

**[0642]** The double-stranded splint adaptors comprised: first splint strands (300) hybridized to second splint strands (400). An exemplary double-stranded splint adaptor is shown in FIG. 2.

**[0643]** The second splint strands (400) comprised: a first sub-region that hybridizes with a third surface primer and a second sub-region that hybridizes with a fourth surface primer (or a complementary sequence thereof). The 5' ends of the second splint strands (400) carried a phosphorylated end. The second splint strands (400) are designed to carry universal sequences that are not found in a commercially-available library preparation kit.

**[0644]** The first splint strands (300) comprised: a first region (320) that hybridizes with a P5 sequence at one end of the linear library molecule; an internal region (310) which hybridized with the second splint strands (400); and a second region (330) that hybridizes with a P7 sequence at the other end of the linear library molecule.

**[0645] EXAMPLE 3: Preparing Covalently Closed Circular Library Molecules**

**[0646]** The annealing mixture from Example 2 was subjected to an enzymatic ligation and phosphorylation reaction by adding to the annealing mixture T7 DNA ligase and T4 polynucleotide kinase with a T4 DNA ligase reaction buffer. The enzymatic mixture was incubated in a thermal cycling apparatus with a heated lid set to 75 degrees C. The thermal cycling apparatus program included: 10 minutes at 37 degrees, 10 minutes at 65 degrees, and hold at 4 degrees. The ligation and phosphorylation reactions generated covalently closed circular library molecules (600) that were hybridized to first splint strands (300).

**[0647]** An enzymatic exonuclease digestion was conducted by adding to the ligation/phosphorylation reaction mixture T7 exonuclease and Thermolabile exonuclease. The exonuclease reaction mixture was incubated in a thermal cycling apparatus which was programed: 10 minutes at 37 degrees C, 2 minutes at 80 degrees C, and hold at 4 degrees C.

**[0648]** The exonuclease reaction mixture was subjected to multiple cycles of clean-up using SPRI SELECT beads (from Beckman Coulter).

**[0649]** The yield of the cleaned preparation of covalently closed circular library molecules (e.g., single stranded molecules) were quantified using Qubit or qPCR. The mean percent circularization recovery are shown in FIG. 24. Lane 1: *Haemophilus influenzae* (38% GC); Lane 2: *E. coli* (51% GC); Lane 3: *Rhodopseudomonas palustris* (65% GC); Lane 4: PhiX; Lane 5: Human; Lane 6: Human exome; Lane 7: Human mRNA.

**[0650] EXAMPLE 4: Rolling Circle Amplification and Sequencing**

**[0651]** The covalently closed circular library molecules from Example 3 were distributed onto a support that was passivated with a low non-specific binding coating in the presence of a high efficiency hybridization buffer, and subjected to on-support rolling circle amplification to generate immobilized concatemers. The mean polony density of the immobilized concatemers is shown in FIG. 25. Lane 1: cell free DNA; Lane 2: *E. coli*; Lane 3: Human; Lane 4: metagenomic DNA; and Lane 5: PhiX.

**[0652] EXAMPLE 5: Sequencing using multivalent molecules and nucleotides**

**[0653]** The concatemers were subjected to recursive two-stage sequencing reactions using fluorescently-labeled multivalent molecules in the first stage and un-labeled nucleotide analogs (e.g., 3' chain terminator blocking group) in the second stage.

**[0654]** The two-stage sequencing reaction was conducted on a flow cell having a plurality of concatemer template molecules immobilized thereon (e.g., immobilized polonies).

**[0655]** The first-stage sequencing reaction was conducted by hybridizing a plurality of a soluble sequencing primers to concatemer template molecules that were immobilized to a flow cells to form immobilized primer-concatemer duplexes. A plurality of a first sequencing polymerase was flowed onto the flow cell (e.g., contacting the immobilized primer-concatemer duplexes) and incubated under a condition suitable to bind the sequencing polymerase to the duplexes to form complexed polymerases. A mixture of fluorescently labeled multivalent molecules (e.g., at different concentrations of about 20-100 nM) was flowed onto the flow cell in the presence of a buffer that included a non-catalytic cation (e.g., strontium, barium and/or calcium) and incubated under conditions suitable to bind complementary nucleotide units of the multivalent molecules to the complexed polymerases to form avidity complexes without polymerase-catalyzed incorporation of the nucleotide units. The fluorescently labeled multivalent molecules were labeled at their cores. The complexed polymerases were washed. An image was obtained of the fluorescently labeled multivalent molecules that remained bound to the complexed polymerases. The first sequencing polymerases and multivalent molecules were removed, while retaining the sequencing primers hybridized to the immobilized concatemers (retained duplexes), by washing with a buffer comprising a detergent.

**[0656]** The first stage sequencing reaction was suitable for forming a plurality of avidity complexes on the concatemer template molecules (e.g., polonies). For example, the first stage sequencing reaction comprised: **(a)** binding a first nucleic acid primer, a first polymerase, and a first multivalent molecule to a first portion of a concatemer template molecule thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent

molecule was bound to the first polymerase; and **(b)** binding a second nucleic acid primer, a second polymerase, and the first multivalent molecule to a second portion of the same concatemer template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the first multivalent molecule was bound to the second polymerase, wherein the first and second binding complexes which included the same multivalent molecule formed a first avidity complex.

**[0657]** The second-stage sequencing reaction was conducted by contacting the retained duplexes with a plurality of second sequencing polymerases to form complexed polymerases. A mixture of non-labeled nucleotide analogs (e.g., 3'-O-methylazido nucleotides) (e.g., at different concentrations of about 1-5  $\mu\text{M}$ ) was added to the complexed polymerases in the presence of a buffer that included a catalytic cation (e.g., magnesium and/or manganese) and incubated under conditions suitable to bind complementary nucleotides to the complexed polymerases and promote polymerase-catalyzed incorporation of the nucleotides to generate a nascent extended sequencing primer. The complexed polymerases were washed. No image was obtained. The incorporated non-labeled nucleotide analogs were reacted with a cleaving reagent that removes the 3' O-methylazido group and generates an extendible 3'OH group.

**[0658]** In an alternative second stage sequencing reaction, a mixture of fluorescently labeled nucleotide analogs (e.g., 3'-O-methylazido nucleotides) (e.g., about 1-5  $\mu\text{M}$ ) was added to the complexed polymerases in the presence of a buffer that included a catalytic cation (e.g., magnesium and/or manganese) and incubated under conditions suitable to bind complementary nucleotides to the complexed polymerases and promote polymerase-catalyzed incorporation of the nucleotides to generate a nascent extended sequencing primer. The complexed polymerases were washed. An image was obtained of the incorporated fluorescently labeled nucleotide analogs as a part of the complexed polymerases. The incorporated fluorescently labeled nucleotide analogs were reacted with a cleaving reagent that removes the 3' O-methylazido group and generates an extendible 3'OH group.

**[0659]** The second sequencing polymerases were removed, while retaining the nascent extended sequencing primers hybridized to the concatemers (retained duplexes), by washing with a buffer comprising a detergent. Recurring sequencing reactions were conducted by performing multiple cycles of first-stage and second-stage sequencing reactions to generate extended forward sequencing primer strands.

**WHAT IS CLAIMED:**

1. A library-splint complex (500) 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 double-stranded splint adaptor (200) which includes a first splint strand (long splint strand (300)) and a second splint strand (short splint strand (400)), wherein the double-stranded splint adaptor (200) includes a double-stranded region and two flanking single-stranded regions, wherein the first splint strand comprises a first region (320), an internal region (310), and a second region (330);  
wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the first region of the first splint strand (320) is hybridized to the at least first left universal adaptor sequence (120) of the library molecule, and wherein a second region of the first splint strand (330) is hybridized to the at least first right universal sequence (130) of the library molecule, thereby circularizing the library molecule to generate a library-splint complex (500).
2. The library-splint complex (500) of claim 1, wherein the nucleic acid library molecule (100) further comprises: a second left universal adaptor sequence (140).
3. The library-splint complex (500) of claim 1, wherein the nucleic acid library molecule (100) further comprises: a second right universal adaptor sequence (150).
4. The library-splint complex (500) of claim 1, wherein the nucleic acid library molecule (100) further comprises: a first left index sequence (160).
5. The library-splint complex (500) of claim 1, wherein the nucleic acid library molecule (100) further comprises: a first right index sequence (170).

6. The library-splint complex (500) of claim 1, wherein the nucleic acid library molecule (100) further comprises: a first left unique identification sequence (180).
7. The library-splint complex (500) of claim 1, wherein the nucleic acid library molecule (100) further comprises: a first right unique identification sequence (190).
8. The library-splint complex (500) of claim 1, wherein the nucleic acid library molecule (100) further comprises any one or any combination of two or more:
  - (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).
9. The library-splint complex (500) of claim 1, 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 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.
10. The library-splint complex (500) of claim 1, 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 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.

11. The library-splint complex (500) of claim 9 or 10, wherein the second splint strand (400) includes at least two sub-regions, the first sub-region comprising a universal binding sequence for a third surface primer, and the second sub-region comprising a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize to (or at least exhibit very little hybridization to) the first and second surface primers.
12. The library-splint complex (500) of claim 11, wherein the second splint strand (400) further comprises an optional third sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN).
13. The library-splint complex (500) of claim 9 or 10, wherein the first splint strand (300) includes an internal region (310) comprising at least two sub-regions, the fourth sub-region comprising a universal binding sequence for a third surface primer and the fourth sub-region hybridizes to the first sub-region of the second splint strand (400), and the fifth sub-region comprising a universal binding sequence for a fourth surface primer and the fifth sub-region hybridizes to the second sub-region of the second splint strand (400), wherein the fourth and fifth sub-regions do not hybridize to (or at least exhibit very little hybridization to) the first and second surface primers.
14. The library-splint complex (500) of claim 13, wherein the first splint strand (300) includes an internal region (310) which further comprises a sixth sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN), wherein the sixth sub-region hybridizes to the third sub-region of the second splint strand (400) .
15. A library-splint complex (500) comprising:
  - a) 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 (120); **(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 (130);
- b) a first splint strand (300) comprising components arranged in a 5' to 3' order: a first region (320); an internal region (310); and a second region (330); and
- c) a second splint strand (400) comprising sub-regions arranged in a 3' to 5' order: a first sub-region having a universal binding sequence for a third surface primer; and a second sub-region having a universal binding sequence for a fourth surface primer; wherein the first splint strand (300) is hybridized to portions of the library molecule (100) thereby circularizing the library molecule to generate a library-splint complex (500), such that the first region (320) of the first splint strand is hybridized to the binding sequence for the first surface primer (120), and the third region (330) of the first splint strand is hybridized to the binding sequence for the second surface primer (130), wherein the second splint strand (400) is hybridized to the internal region (310) of the first splint strand (300), wherein the library-splint complex (500) comprises a first nick between the 5' end of the library molecule and the 3' end of the second splint strand, wherein the library-splint complex (500) comprises a second nick between the 5' end of the second splint strand and the 3' end of the library molecule, and wherein the first and second nicks are enzymatically ligatable.
16. The library-splint complex (500) of any of the preceding claims, further comprising a plurality of library-splint complexes (500), wherein the sequence of interest (110) of individual library-splint complexes in the plurality comprise the same sequence of interest or different sequences of interest.
17. A reaction mixture comprising the plurality of library-splint complexes (500) of claim 16 and T4 polynucleotide kinase.
18. A reaction mixture comprising the plurality of library-splint complexes (500) of claim 16 and a ligase enzyme.

19. A reaction mixture comprising the plurality of library-splint complexes (500) of claim 16, T4 polynucleotide kinase, and a ligase enzyme.
20. The reaction mixture of claim 18 or 19, wherein the ligase enzyme comprises T7 DNA ligase, T3 ligase, T4 ligase, or Taq ligase.
21. A covalently closed circular library molecule (600) comprising: a sequence of interest (110), at least a first left universal adaptor sequence (120), at least a first right universal adaptor sequence (130), and a second splint strand sequence (400).
22. The covalently closed circular library molecule (600) of claim 21, further comprising: a second left universal adaptor sequence (140).
23. The covalently closed circular library molecule (600) of claim 21, further comprising: a second right universal adaptor sequence (150).
24. The covalently closed circular library molecule (600) of claim 21, further comprising: a first left index sequence (160).
25. The covalently closed circular library molecule (600) of claim 21, further comprising: a first right index sequence (170).
26. The covalently closed circular library molecule (600) of claim 21, further comprising: a first left unique identification sequence (180).
27. The covalently closed circular library molecule (600) of claim 21, further comprising: a first right unique identification sequence (190).
28. The covalently closed circular library molecule (600) of claim 21, further comprising any one or any combination of two or more:
  - (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).
29. The covalently closed circular library molecule (600) of claim 21, 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 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.
30. The covalently closed circular library molecule (600) of claim 21, 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 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.
31. The covalently closed circular library molecule (600) of any one of claims 21-30, which is hybridized to a first splint strand (long splint strand (300)) which comprises a first region (320), an internal region (310), and a second region (330), wherein the second splint strand sequence (400) of the covalently closed circular library molecule is hybridized to the internal region (310) of the first splint strand, wherein the first region of the first splint strand (320) is hybridized to the at least first left universal adaptor sequence (120) of the library molecule, and wherein a second region of the first splint strand (330) is hybridized to the at least first right universal sequence (130) of the library molecule.

32. The covalently closed circular library molecule (600) of claim 31, wherein the second splint strand (400) includes at least two sub-regions, the first sub-region comprising a universal binding sequence for a third surface primer, and the second sub-region comprising a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize to (or at least exhibit very little hybridization to) the first and second surface primers.
33. The covalently closed circular library molecule (600) of claim 32, wherein the second splint strand (400) further comprises an optional third sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN).
34. The covalently closed circular library molecule (600) of claim 31, wherein the first splint strand (300) includes an internal region (310) comprising at least two sub-regions, the fourth sub-region comprising a universal binding sequence for a third surface primer and the fourth sub-region hybridizes to the first sub-region of the second splint strand (400), and the fifth sub-region comprising a universal binding sequence for a fourth surface primer and the fifth sub-region hybridizes to the second sub-region of the second splint strand (400), wherein the fourth and fifth sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers.
35. The covalently closed circular library molecule (600) of claim 34, wherein the first splint strand (300) includes an internal region (310) which further comprises a sixth sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN), wherein the sixth sub-region hybridizes to the third sub-region of the second splint strand (400) .
36. A single-stranded covalently closed circular molecule (600) comprising: **(i)** a first left universal adaptor sequence (120) having a binding sequence for a first surface primer (120); **(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; **(v)** a first right universal adaptor sequence (130) having a binding sequence for a second

- surface primer (130); and (vi) a second splint strand (400), wherein the covalently closed circular molecule (600) is optionally hybridized to the first splint strand (300).
37. The covalently closed circular library molecule (600) of any one of claims 21-36, further comprising a plurality of covalently closed circular library molecules (600), wherein the sequence of interest (110) of individual covalently closed circular library molecules (600) in the plurality comprise the same sequence of interest or different sequences of interest.
38. A reaction mixture comprising the plurality of plurality of covalently closed circular library molecules (600) of claim 36, wherein individual covalently closed circular library molecules (600) are hybridized to a first splint strand (300).
39. The reaction mixture of claim 38, comprising the plurality of covalently closed circular library molecules (600) and at least one exonuclease enzyme.
40. The reaction mixture of claim 38, wherein the exonuclease enzyme comprises exonuclease I, thermolabile exonuclease I, or T7 exonuclease.
41. A kit, comprising: a double-stranded splint adaptor (200) which includes
- (i) a first splint strand (long splint strand (300)) and
  - (ii) a second splint strand (short splint strand (400)), wherein the first splint strand (300) can hybridize to the second splint strand (400) to form a double-stranded region and two flanking single-stranded regions, wherein the first splint strand (300) comprises a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) can hybridize to the second splint strand (400).
42. The kit of claim 41, wherein the first splint strand (300) and the second splint strand (400) are hybridized to each other to form a double-stranded splint adaptor (200) having a double-stranded region and two flanking single-stranded regions, and wherein the internal region of the first splint strand (310) can hybridize to the second splint strand (400).

43. The kit of claim 41 or 42 for the use in circularizing a single-stranded nucleic acid library molecule (100) having 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).
44. The kit of claim 43, wherein the first region of the first splint strand (320) can hybridize to at least first left universal adaptor sequence (120) of the library molecule (100), and wherein a second region of the first splint strand (330) can hybridize to the at least first right universal sequence (130) of the library molecule.
45. The kit of claim 43, wherein the at least first left universal adaptor sequence (120) of the library molecule (100) 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.
46. The kit of claim 43, wherein the at least first right universal adaptor sequence (130) of the library molecule (100) 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.
47. The kit of claim 43, wherein the second splint strand (400) includes at least two sub-regions, the first sub-region comprising a universal binding sequence for a third surface primer, and the second sub-region comprising a universal binding sequence

- for a fourth surface primer, wherein the first and second sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers.
48. The kit of claim 47, wherein the second splint strand (400) further comprises an optional third sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN).
49. The kit of claim 43, wherein the first splint strand (300) includes an internal region (310) comprising at least two sub-regions, the fourth sub-region comprising a universal binding sequence for a third surface primer and the fourth sub-region hybridizes to the first sub-region of the second splint strand (400), and the fifth sub-region comprising a universal binding sequence for a fourth surface primer and the fifth sub-region hybridizes to the second sub-region of the second splint strand (400), wherein the fourth and fifth sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers.
50. The kit of claim 49, wherein the first splint strand (300) includes an internal region (310) which further comprises a sixth sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN), wherein the sixth sub-region hybridizes to the third sub-region of the second splint strand (400) .
51. The kit of any one of claims 41-50, further comprising T4 polynucleotide kinase.
52. The kit of any one of claims 41-50, further comprising a ligase enzyme.
53. The kit of any one of claims 41-50, further comprising T4 polynucleotide kinase and a ligase enzyme.
54. The kit of claim 52 or 53, wherein the ligase enzyme comprises T7 DNA ligase, T3 ligase, T4 ligase, or Taq ligase.
55. A method for forming a plurality of library-splint complexes (500) comprising:

- a) providing a plurality of double-stranded splint adaptors wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the double-stranded splint adaptor includes a double-stranded region and two flanking single-stranded regions, wherein the first splint strand comprises a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400); and
  - b) hybridizing the plurality of double-stranded splint adaptors with a plurality of single-stranded nucleic acid library molecules (100) wherein individual library molecules include 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), wherein the hybridizing is conducted under a condition suitable to hybridize the first region of the first splint strand (320) to the at least first left universal adaptor sequence (120) of the library molecule, and the condition is suitable to hybridize the second region of the first splint strand (330) to the at least first right universal sequence (130) of the library molecule, thereby circularizing the plurality of library molecules to form a plurality of library-splint complexes (500).
56. The method of claim 55, wherein individual library-splint complexes (500) in the plurality comprise a first nick between the 5' end of the library molecule and the 3' end of the second splint strand, and wherein individual library-splint complexes (500) in the plurality comprise a second nick between the 5' end of the second splint strand and the 3' end of the library molecule.
57. The method of claim 55, wherein individual nucleic acid library molecules (100) in the plurality further comprise: a second left universal adaptor sequence (140).
58. The method of claim 55, wherein individual nucleic acid library molecules (100) in the plurality further comprise: a second right universal adaptor sequence (150).
59. The method of claim 55, wherein individual nucleic acid library molecules (100) in the plurality further comprise: a first left index sequence (160).

60. The method of claim 55, wherein individual nucleic acid library molecules (100) in the plurality further comprise: a first right index sequence (170).
61. The method of claim 55, wherein individual nucleic acid library molecules (100) in the plurality further comprise: a first left unique identification sequence (180).
62. The method of claim 55, wherein individual nucleic acid library molecules (100) in the plurality further comprise: a first right unique identification sequence (190).
63. The method of claim 55, wherein individual nucleic acid library molecules (100) in the plurality further comprise 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).
64. The method of claim 55 or 63, wherein the first and/or the second left universal adaptor sequence 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.
65. The method of claim 55 or 63, wherein the first and/or the second right universal adaptor sequence 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.
66. The method of claim 64 or 65, wherein the second splint strand (400) includes at least two sub-regions, the first sub-region comprising a universal binding sequence for a third surface primer, and the second sub-region comprising a universal binding sequence for a fourth surface primer, wherein the first and second sub-regions do not hybridize to (or at least exhibit very little hybridization to) the first and second surface primers.
67. The method of claim 66, wherein the second splint strand (400) further comprises an optional third sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN).
68. The method of claim 64 or 65, wherein the first splint strand (300) includes an internal region (310) comprising at least two sub-regions, the fourth sub-region comprising a universal binding sequence for a third surface primer and the fourth sub-region hybridizes to the first sub-region of the second splint strand (400), and the fifth sub-region comprising a universal binding sequence for a fourth surface primer and the fifth sub-region hybridizes to the second sub-region of the second splint strand (400), wherein the fourth and fifth sub-regions do not hybridize (or at least exhibit very little hybridization to) the first and second surface primers.
69. The method of claim 68, wherein the first splint strand (300) includes an internal region (310) which further comprises a sixth sub-region which comprises a sample index sequence having 5-20 bases and/or a unique identification sequence having 2-10 or more bases (e.g., NN), wherein the sixth sub-region hybridizes to the third sub-region of the second splint strand (400) .
70. A method for forming a plurality of library-splint complexes (500) comprising:
- a) providing a plurality of double-stranded splint adaptors (200) wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the first splint strand

(300) comprises regions arranged in a 5' to 3' order a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the second splint strand comprises regions arranged in a 5' to 3' order **(i)** a second sub-region having a universal binding sequence for a fourth surface primer, and **(ii)** a first sub-region having a universal binding sequence for a third surface primer; and

- b) hybridizing the plurality of double-stranded splint adaptors with a plurality of single-stranded nucleic acid library molecules (100) wherein individual library molecules 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 (120); **(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 (130),

wherein the hybridizing is conducted under a condition suitable to hybridize portions of the first splint strand (300) to portions of the library molecule (100) thereby circularizing the library molecule to generate a library-splint complex (500), such that the first region (320) of the first splint strand is hybridized to the binding sequence for the first surface primer (120), and the third region (330) of the first splint strand is hybridized to the binding sequence for the second surface primer (130), wherein the library-splint complex (500) comprises a first nick between the 5' end of the library molecule and the 3' end of the second splint strand (300), wherein the library-splint complex (500) comprises a second nick between the 5' end of the second splint strand (300) and the 3' end of the library molecule (100), and wherein the first and second nicks are enzymatically ligatable.

71. The method of claim 70, wherein the plurality of single-stranded nucleic acid library molecules (100) further comprise a first left index sequence (160) and/or a first right index sequence (170).

72. The method of claim 70, 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).
73. The method of any one of claims 55-72, further comprising: contacting the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a T4 polynucleotide kinase enzyme under a condition suitable to phosphorylate the 5' ends of the plurality of double-stranded splint adaptors (200) and/or the plurality of single-stranded nucleic acid library molecules (100).
74. The method of any one of claims 55-72, further comprising: contacting the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a ligase, under a condition suitable to enzymatically ligate the first and second nicks, thereby generating a plurality of covalently closed circular library molecules (600) each hybridized to the first splint strand (300).
75. The method of any one of claims 55-72, further comprising conducting the sequential steps of:
- a) contacting the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a T4 polynucleotide kinase enzyme under a condition suitable to phosphorylate the 5' ends of the plurality of double-stranded splint adaptors (200) and the plurality of single-stranded nucleic acid library molecules (100); and
  - b) contacting the phosphorylated double-stranded splint adaptors (200) and the phosphorylated single-stranded nucleic acid library molecules (100) with a ligase under a condition suitable to enzymatically ligate the first and second nicks, thereby generating a plurality of covalently closed circular library molecules (600) each hybridized to the first splint strand (300).
76. The method of any one of claims 55-72, further comprising conducting the essentially simultaneous steps of contacting the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a
- (i) T4 polynucleotide kinase enzyme and
  - (ii) a ligase enzyme, under a condition suitable

to phosphorylate the 5' ends of the plurality of double-stranded splint adaptors (200) and the plurality of single-stranded nucleic acid library molecules (100), and the conditions are suitable to enzymatically ligate the first and second nicks, thereby generating a plurality of covalently closed circular library molecules (600) each hybridized to the first splint strand (300).

77. The method of claim 74, , further comprising: contacting the plurality of covalently closed circular library molecules (600) with at least one exonuclease enzyme to remove the plurality of first splint strands (300) and retaining the plurality of covalently closed circular library molecules (600).
78. The method of claim 77, wherein the at least one exonuclease enzyme comprises exonuclease I, thermolabile exonuclease I and/or T7 exonuclease.
79. The method of claim 77, wherein individual covalently closed circular library molecules (600) in the plurality comprise a second splint strand region (400) which includes a universal binding sequence for a third surface primer, and wherein the method further comprises: distributing the retained plurality of covalently closed circular library molecules (600) onto a support having a plurality of the third surface primers immobilized on the support, under a condition suitable for hybridizing individual covalently closed circular library molecules (600) to individual immobilized third surface primers thereby immobilizing the plurality of covalently closed circular library molecules (600).
80. The method of claim 79, further comprising: contacting the plurality of immobilized covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of third surface primers as immobilized amplification primers and the plurality of covalently closed circular library molecules (600) as template molecules, thereby generating a plurality of immobilized nucleic acid concatemer molecules.
81. The method of claim 80, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

82. The method of claim 77, wherein the first (120) or the second (140) left universal adaptor sequence of individual library molecules in the plurality comprise a universal binding sequence for a forward amplification primer, and wherein the method further comprises:
- a) hybridizing a plurality of soluble forward amplification primers to the first or second left universal adaptor sequence which comprise the universal binding sequence for a forward amplification primer; and
  - b) conducting a rolling circle amplification reaction thereby generating a plurality of nucleic acid concatemer molecules.
83. The method of claim 77, wherein the first (130) or the second (150) right universal adaptor sequence of individual library molecules in the plurality comprise a universal binding sequence for a forward amplification primer, and wherein the method further comprises:
- a) hybridizing a plurality of soluble forward amplification primers to the first or second right universal adaptor sequence which comprise the universal binding sequence for a forward amplification primer; and
  - b) conducting a rolling circle amplification reaction thereby generating a plurality of nucleic acid concatemer molecules each hybridized to a covalently closed circular library molecule (600).
84. The method of claim 82 or 83, wherein individual concatemer molecules in the plurality comprise a second splint strand region (400) having a universal binding sequence for third surface primer, wherein individual concatemer molecules are hybridized to a covalently closed circular library molecule (600), and wherein the method further comprises: distributing the plurality of concatemer molecules wherein onto a support having a plurality of the third surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized third surface primers thereby immobilizing the plurality of concatemer molecules.
85. The method of claim 84, further comprising: contacting the immobilized plurality of concatemer molecules (which are hybridized to a covalently closed circular library

molecule (600)) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules.

86. The method of claim 85, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.
87. The method of any one of claims 74-76, wherein individual covalently closed circular library molecules (600) in the plurality are hybridized to a first splint strand (300), and wherein the method further comprises: contacting the plurality of covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides under a condition suitable for conducting a rolling circle amplification reaction using the first splint strand (300) as an amplification primer thereby generating a plurality of concatemer molecules.
88. The method of claim 87, wherein individual concatemer molecules in the plurality comprise a second splint strand region (400) which includes a universal binding sequence for third surface primer, and wherein the method further comprises: distributing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600) onto a support having a plurality of the third surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized third surface primers thereby immobilizing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600).
89. The method of claim 88, further comprising: contacting the plurality of immobilized concatemer molecules with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules.

90. The method of claim 89, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.
91. The method of any one of claims 80, 85 or 89, wherein the support further comprises a plurality of the fourth surface primers immobilized on the support.
92. The method of claim 91, wherein the plurality of immobilized nucleic acid concatemer molecules comprise at least two second splint strand regions (400) each having a universal binding sequence for the third and fourth surface primer, and wherein the method further comprises: incubating the plurality of immobilized nucleic acid concatemer molecules under a condition suitable for hybridizing at least one second splint strand region (400) of at least one concatemer molecule to an immobilized fourth surface primer thereby pinning down at least one portion of the immobilized concatemer molecule to the support.
93. The method of any one of claims 80, 85, 89 or 92, further comprising: sequencing the plurality of immobilized concatemers, the method comprising:
- a) contacting the plurality of immobilized concatemer molecules with (i) a plurality of sequencing polymerases and (ii) a plurality of the soluble 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 concatemer molecule hybridized to a soluble 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 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.

94. The method of any one of claims 80, 85, 89 or 92, further comprising: sequencing the plurality of immobilized concatemers, the method comprising:
- a) contacting the plurality of immobilized concatemer molecules with (i) a plurality of sequencing polymerases and (ii) a plurality of the soluble sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each 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 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.
95. The method of claim 94, further comprising:
- e) dissociating the plurality of multivalent-complexed polymerases and removing the plurality of first sequencing polymerases and their bound multivalent molecules, and retaining the plurality of nucleic acid duplexes;
  - f) contacting the plurality of the retained nucleic acid duplexes of step (e) with a plurality of second sequencing polymerases, wherein the contacting is conducted under a condition suitable for binding the plurality of second

- sequencing polymerases to the plurality of the retained nucleic acid duplexes, thereby forming a plurality of second complexed polymerases each comprising a second sequencing polymerase bound to a retained nucleic acid duplex;
- g) contacting the plurality of second complexed polymerases with a plurality of nucleotides, wherein the contacting is conducted under a condition suitable for binding complementary nucleotides from the plurality of nucleotides to at least two of the second complexed polymerases of step (f) thereby forming a plurality of nucleotide-complexed polymerases and the condition is suitable for promoting incorporation of the bound complementary nucleotides into the sequencing primers of the nucleotide-complexed polymerases.
96. The method of claim 95, wherein the plurality of nucleotides includes at least one nucleotide analog labeled with a fluorophore and having a removable chain terminating moiety at the sugar 3' position.
97. The method of claim 95, further comprising:
- h) detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.
98. The method of claim 95, 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.
99. A method for forming at least one avidity complex, comprising:
- a) binding a first universal nucleic acid primer, a first DNA polymerase, and a first multivalent molecule to a first portion of any of the concatemer molecules of any one of claims 80, 85, 89 or 92, thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first DNA polymerase; and

- b) binding a second universal nucleic acid primer, a second DNA polymerase, and the first multivalent molecule to a second portion of the same concatemer template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the first multivalent molecule binds to the second DNA polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms an avidity complex, wherein the first multivalent molecule comprises a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit, and wherein the concatemer molecule comprises two or more tandem repeat sequences of a sequence of interest (110) and a universal primer binding site that binds the first and second universal nucleic acid primers.

100. A method for sequencing by forming at least one avidity complex, comprising:

- a) binding a first universal nucleic acid primer, a first DNA polymerase, and a first multivalent molecule to a first portion of any of the concatemer molecules of any one of claims 80, 85, 89 or 92, thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first DNA polymerase;
- b) binding a second universal nucleic acid primer, a second DNA polymerase, and the first multivalent molecule to a second portion of the same concatemer template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the first multivalent molecule binds to the second DNA polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms an avidity complex, wherein the first multivalent molecule comprises a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit, wherein the concatemer molecule comprises two or more tandem repeat sequences of a sequence of interest (110) and a universal primer binding site that binds the first and second universal nucleic acid primers, and 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;
- c) detecting the first and second binding complexes on the same concatemer template molecule, and

- d) identifying the first nucleotide unit in the first binding complex thereby determining the sequence of the first portion of the concatemer template molecule, and identifying the second nucleotide unit in the second binding complex thereby determining the sequence of the second portion of the concatemer template molecule.

101. A method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources, comprising:

- a) providing two or more populations of single-stranded nucleic acid library molecules (100), each population of library molecules (100) contained in a separate compartment, wherein the nucleic acid library molecules in a given population comprise
  - **(i)** a first left universal adaptor sequence (120) having a binding sequence for a first surface primer,
  - **(ii)** a second left universal adaptor sequence (140) having a binding sequence for a first sequencing primer;
  - **(iii)** a sequence of interest (110) isolated from a sample source,
  - **(iv)** a second right universal adaptor sequence (150) having a binding sequence for a second sequencing primer;
  - **(v)** a first right universal adaptor sequence (130) having a binding sequence for a second surface primer, and
  - **(vii)** a first left index sequence (160) and/or a first right index sequence (170), wherein the sequences of the left (160) and/or right (170) indexes separately or in combination identify the sample source from which the sequences of interest (110) have been isolated;
- b) providing a plurality of double-stranded splint adaptors (200) wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the first splint strand (300) comprises regions arranged in a 5' to 3' order a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the second splint strand comprises regions arranged in a 5' to 3' order **(i)** a second sub-region having a universal binding sequence for a

- fourth surface primer, and (ii) a first sub-region having a universal binding sequence for a third surface primer;
- c) contacting in the separate compartments the population of single-stranded nucleic acid library molecules (100) with a plurality of double-stranded splint adaptors (200), wherein the contacting is conducted under a condition suitable to hybridize portions of the first splint strand (300) to portions of the library molecules (100) thereby circularizing the library molecules to generate a population of library-splint complexes (500), such that the first region (320) of an individual first splint strand is hybridized to the binding sequence for the first surface primer (120) of an individual library molecule (100), and the third region (330) of the individual first splint strand is hybridized to the binding sequence for the second surface primer (130) of the individual library molecule (100), wherein each of the library-splint complexes (500) comprise a first nick between the 5' end of the library molecule and the 3' end of the second splint strand (300), wherein each of the library-splint complexes (500) comprises a second nick between the 5' end of the second splint strand (300) and the 3' end of the library molecule (100), and wherein the first and second nicks are enzymatically ligatable;
  - d) contacting in the separate compartments the populations of library-splint complexes (500) with a ligase, under a condition suitable to enzymatically ligate the first and second nicks, thereby generating two or more populations of covalently closed circular library molecules (600) each hybridized to the first splint strand (300); and
  - e) pooling together the two or more populations of covalently closed circular library molecules (600) from the separate compartments to generate a multiplex mixture of covalently closed circular library molecules (600) which comprise the multiplex mixture of sequences-of-interest isolated from a plurality of sample sources.

102. The method of claim 101, wherein individual covalently closed circular library molecules (600) in the multiplex mixture of step (e) comprise a second splint strand region (400) which includes a universal binding sequence for a third surface primer, and wherein the method further comprises:

- a) distributing the multiplex mixture of covalently closed circular library molecules (600) onto a support having a plurality of the third surface primers immobilized on the support, under a condition suitable for hybridizing individual covalently closed circular library molecules (600) to individual immobilized third surface primers thereby immobilizing the plurality of covalently closed circular library molecules (600); and
- b) contacting the multiplex mixture of immobilized covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of third surface primers as immobilized amplification primers and the plurality of covalently closed circular library molecules (600) as template molecules, thereby generating a plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

103. The method of claim 101, further comprising: conducting a rolling circle amplification reaction by:

- a) contacting the two or more populations of covalently closed circular library molecules (600) that are hybridized to the first splint strand (300) of step (d) or (e) with at least one exonuclease enzyme under a condition suitable to remove the plurality of first splint strands (300) and retaining the population of covalently closed circular library molecules (600); and
- b) contacting the retained two or more populations of covalently closed circular library molecules (600) with a plurality of soluble amplification primers, a plurality of strand-displacing polymerases, and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction thereby generating a plurality of nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

104. The method of claim 103, further comprising:

- c) distributing the plurality of concatemer molecules onto a support having a plurality of the third surface primers immobilized thereon, under a condition

suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized third surface primers thereby immobilizing the plurality of concatemer molecules; and

- d) contacting the immobilized plurality of concatemer molecules (which are hybridized to a covalently closed circular library molecule (600)) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

105. The method of claim 101, further comprising: conducting a rolling circle amplification reaction by contacting the two or more populations of covalently closed circular library molecules (600) each hybridized to the first splint strand (300) of step (d) or (e) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction thereby generating a plurality of concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

106. The method of claim 105, further comprising:

- a) distributing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600) onto a support having a plurality of the third surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized third surface primers thereby immobilizing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600); and
- b) contacting the plurality of immobilized concatemer molecules with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized

nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

107. The method of any one of claims 102, 104 or 106, further comprising: sequencing the insert region (110) and the first left index sequence (160) and/or the first right index sequence (170) of the plurality of immobilized nucleic acid concatemer molecules, wherein the sequences of the insert region (110) along with the first left index sequence (160) and/or first right index sequence (170) can be used to identify the sample source of the sequence of interest.
108. The method of claim 107, wherein the sequencing reaction comprises:
- binding the plurality of immobilized nucleic acid concatemer molecules with a plurality of sequencing primers, a plurality of sequencing polymerases, and a plurality of detectably labeled nucleotide analogs each comprising a 2' or 3' chain terminating moiety, under a condition suitable for incorporating the detectably labeled nucleotide analogs into the 3' end of the sequencing primers;
  - detecting the bound detectably labeled nucleotide analog; and
  - identifying the nucleobase of the detectably labeled nucleotide analog.
109. The method of claim 107, wherein the sequencing reaction employs a two-stage sequencing reaction, wherein the first stage comprises:
- binding the plurality of immobilized nucleic acid concatemer molecules with a plurality of sequencing primers, a plurality of a first sequencing polymerase, and a plurality of detectably labeled multivalent molecules, under a condition suitable to bind individual concatemer molecules to a sequencing primer, a first sequencing polymerase, and a detectably labeled multivalent molecule (e.g., forming a plurality of multivalent-complexed polymerases);
  - detecting the bound detectably labeled multivalent molecule; and
  - identifying the nucleobase of the detectably labeled multivalent molecule.
110. The method of claim 109, wherein the binding of the detectably labeled multivalent molecules to the plurality of immobilized nucleic acid concatemer molecules forms at least one avidity complex according to the method of claim 99 or 100.

111. The method of claim 109, further comprising conducting a second stage which comprises:

- d) removing the plurality of first sequencing polymerases and the plurality of bound multivalent molecules from the plurality of immobilized nucleic acid concatemer molecules and retaining the immobilized concatemer molecules each hybridized to a sequencing primer (nucleic acid duplex); and
- e) contacting the retained immobilized concatemer molecules and hybridized sequencing primers with a plurality of second polymerases and a plurality of nucleotides under a condition suitable for incorporating the nucleotides into the 3' end of the sequencing primers.

112. A method for preparing a multiplex mixture of sequences-of-interest isolated from a plurality of sample sources, comprising:

- a) providing two or more populations of single-stranded nucleic acid library molecules (100), each population of library molecules (100), each population of single-stranded nucleic acid library molecules is contained in a separate compartment, wherein the nucleic acid library molecules in a given population comprise
  - **(i)** a first left universal adaptor sequence (120) having a binding sequence for a first surface primer,
  - **(ii)** a second left universal adaptor sequence (140) having a binding sequence for a first sequencing primer;
  - **(iii)** a sequence of interest (110) isolated from a sample source,
  - **(iv)** a second right universal adaptor sequence (150) having a binding sequence for a second sequencing primer;
  - **(v)** a first right universal adaptor sequence (130) having a binding sequence for a second surface primer, and
  - **(vi)** a first left index sequence (160) and/or a first right index sequence (170), wherein the sequences of the left (160) and/or right (170) indexes separately or in combination identify the sample source from which the sequences of interest (110) are isolated;

- b) pooling together the two or more populations of single-stranded nucleic acid library molecules (100) into the same compartment;
- c) adding to the compartment of step (b) a plurality of double-stranded splint adaptors (200) wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the first splint strand (300) comprises regions arranged in a 5' to 3' order a first region (320), an internal region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the second splint strand comprises regions arranged in a 5' to 3' order of **(i)** a second sub-region having a universal binding sequence for a fourth surface primer, and **(ii)** a first sub-region having a universal binding sequence for a third surface primer;
- d) incubating the population of single-stranded nucleic acid library molecules (100) and the plurality of double-stranded splint adaptors (200) which are in the same compartment of step (c) under a condition suitable to hybridize portions of the first splint strand (300) to portions of the library molecules (100) thereby circularizing the library molecules to generate a population of library-splint complexes (500), such that the first region (320) of an individual first splint strand is hybridized to the binding sequence for the first surface primer (120) of an individual library molecule (100), and the third region (330) of the individual first splint strand is hybridized to the binding sequence for the second surface primer (130) of the individual library molecule (100), wherein each of the library-splint complexes (500) comprise a first nick between the 5' end of the library molecule and the 3' end of the second splint strand (300), wherein each of the library-splint complexes (500) comprises a second nick between the 5' end of the second splint strand (300) and the 3' end of the library molecule (100), and wherein the first and second nicks are enzymatically ligatable; and
- e) contacting the populations of library-splint complexes (500) in the compartment of step (d) with a ligase, under a condition suitable to enzymatically ligate the first and second nicks, thereby generating a population of covalently closed circular library molecules (600) each hybridized to the first splint strand (300), thereby generating a multiplex mixture of covalently closed circular library molecules (600) which comprise the multiplex mixture of sequences-of-interest isolated from a plurality of sample sources.

113. The method of claim 112, wherein individual covalently closed circular library molecules (600) in the multiplex mixture of step (e) comprise a second splint strand region (400) which includes a universal binding sequence for a third surface primer, and wherein the method further comprises:
- a) distributing the plurality of covalently closed circular library molecules (600) onto a support having a plurality of the third surface primers immobilized on the support, under a condition suitable for hybridizing individual covalently closed circular library molecules (600) to individual immobilized third surface primers thereby immobilizing the plurality of covalently closed circular library molecules (600); and
  - b) contacting the plurality of immobilized covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of third surface primers as immobilized amplification primers and the plurality of covalently closed circular library molecules (600) as template molecules, thereby generating a plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.
114. The method of claim 112, further comprising: conducting a rolling circle amplification reaction by:
- a) contacting the population of covalently closed circular library molecules (600) that are hybridized to the first splint strand (300) of step (d) or (e) with at least one exonuclease enzyme under a condition suitable to remove the plurality of first splint strands (300) and retaining the population of covalently closed circular library molecules (600);
  - b) contacting the retained population of covalently closed circular library molecules (600) with a plurality of soluble amplification primers, a plurality of strand-displacing polymerases, and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction thereby generating a plurality of nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

115. The method of claim 114, further comprising:

- c) distributing the plurality of concatemer molecules onto a support having a plurality of the third surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized third surface primers thereby immobilizing the plurality of concatemer molecules; and
- d) contacting the immobilized plurality of concatemer molecules (which are hybridized to a covalently closed circular library molecule (600)) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

116. The method of claim 112, further comprising: conducting a rolling circle amplification reaction by contacting the population of covalently closed circular library molecules (600) each hybridized to the first splint strand (300) of step (d) or (e) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct a rolling circle amplification reaction thereby generating a plurality of concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

117. The method of claim 116, further comprising:

- c) distributing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600) onto a support having a plurality of the third surface primers immobilized thereon, under a condition suitable for hybridizing at least a portion of the concatemers to at least a portion of the plurality of the immobilized third surface primers thereby immobilizing the plurality of concatemer molecules which are hybridized to a covalently closed circular library molecule (600); and
- d) contacting the plurality of immobilized concatemer molecules with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a

condition suitable to conduct a rolling circle amplification reaction on the support using the plurality of covalently closed circular library molecules (600) as template molecules, thereby extending the plurality of immobilized nucleic acid concatemer molecules, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.

118. The method of any one of claims 113, 115 or 117, further comprising: sequencing the insert region (110) and the first left index sequence (160) and/or the first right index sequence (170) of the plurality of immobilized nucleic acid concatemer molecules, wherein the sequences of the insert region (110) along with the first left index sequence (160) and/or first right index sequence (170) can be used to identify the sample source of the sequence of interest.
119. The method of claim 118, wherein the sequencing reaction comprises:
- binding the plurality of immobilized nucleic acid concatemer molecules with a plurality of sequencing primers, a plurality of sequencing polymerases, and a plurality of detectably labeled nucleotide analogs each comprising a 2' or 3' chain terminating moiety, under a condition suitable for incorporating the detectably labeled nucleotide analogs into the 3' end of the sequencing primers;
  - detecting the bound detectably labeled nucleotide analog; and
  - identifying the nucleobase of the detectably labeled nucleotide analog.
120. The method of claim 118, wherein the sequencing reaction employs a two-stage sequencing reaction, wherein the first stage comprises:
- binding the plurality of immobilized nucleic acid concatemer molecules with a plurality of sequencing primers, a plurality of a first sequencing polymerase, and a plurality of detectably labeled multivalent molecules, under a condition suitable to bind individual concatemer molecules to a sequencing primer, a first sequencing polymerase, and a detectably labeled multivalent molecule (e.g., forming a plurality of multivalent-complexed polymerases);
  - detecting the bound detectably labeled multivalent molecule; and
  - identifying the nucleobase of the detectably labeled multivalent molecule.

121. The method of claim 120, wherein the binding of the detectably labeled multivalent molecules to the plurality of immobilized nucleic acid concatemer molecules forms at least one avidity complex according to the method of claim 99 or 100.
122. The method of claim 120, further comprising conducting a second stage which comprises:
- d) removing the plurality of first sequencing polymerases and the plurality of bound multivalent molecules from the plurality of immobilized nucleic acid concatemer molecules and retaining the immobilized concatemer molecules each hybridized to a sequencing primer (nucleic acid duplex); and
  - e) contacting the retained immobilized concatemer molecules and hybridized sequencing primers with a plurality of second polymerases and a plurality of nucleotides under a condition suitable for incorporating the nucleotides into the 3' end of the sequencing primers.
123. A method for appending additional universal adaptor sequencing to nucleic acid library molecules, comprising:
- a) providing a plurality of nucleic acid library molecules (100) each comprising sequences arranged in a 5' to 3' order:
    - (i) a first left universal adaptor sequence (120),
    - (ii) a second left universal adaptor sequence (140),
    - (iii) a sequence of interest (110),
    - (iv) a second right universal adaptor sequence (150), and
    - (v) a first right universal adaptor sequence (130);
  - b) hybridizing individual library molecules of step (a) with a first tailed primer and conducting a first primer extension reaction (or first PCR), wherein the first tailed primers comprise a 5' region carrying a third left universal adaptor sequence and a 3' region that is complementary to at least a portion of the first left universal adaptor sequence (120) of the library molecule (100), wherein the first primer extension reaction (or first PCR) generates a plurality of first modified library molecules each appended at one end with the third left universal adaptor sequence, wherein the third left universal adaptor sequence of the first tailed primers comprise

- a universal binding sequence for a forward or reverse sequencing primer,
  - a universal binding sequence for a first or second surface primer,
  - a universal binding sequence for a forward or reverse amplification primer and/or
  - a universal binding sequence for a compaction oligonucleotide; and
- c) hybridizing individual first modified library molecules of step (a) with a second tailed primer and conducting a second primer extension reaction (or second PCR), wherein the second tailed primers comprise a 5' region carrying a third right universal adaptor sequence and a 3' region that is complementary to at least a portion of the first right universal adaptor sequence (130) of the first modified library molecule, wherein the second primer extension reaction (or second PCR) generates a plurality of second modified library molecules each appended at one end with the third right universal adaptor sequence, wherein the third right universal adaptor sequence of the second tailed primers comprise
- a universal binding sequence for a forward or reverse sequencing primer,
  - a universal binding sequence for a first or second surface primer,
  - a universal binding sequence for a forward or reverse amplification primer and/or
  - a universal binding sequence for a compaction oligonucleotide.

124. The method of claim 123, wherein the nucleic acid library molecules each comprise sequences arranged in a 5' to 3' order:

- (i) a first left universal adaptor sequence (120) having a binding sequence for a first surface primer,
- (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.

125. The method of claim 123, further comprising:

- d) hybridizing individual second modified library molecules of step (c) with a third tailed primer and conducting a third primer extension reaction (or third PCR), wherein the third tailed primers comprise a 5' region carrying a fourth left universal adaptor sequence and a 3' region that is complementary to at least a portion of the third left universal adaptor sequence of the second modified library molecule, wherein the third primer extension reaction (or third PCR) generates a plurality of third modified library molecules each appended at one end with the fourth left universal adaptor sequence, wherein the fourth left universal adaptor sequence of the third tailed primers comprise
  - a universal binding sequence for a forward or reverse sequencing primer,
  - a universal binding sequence for a first or second surface primer,
  - a universal binding sequence for a forward or reverse amplification primer and/or
  - a universal binding sequence for a compaction oligonucleotide; and
- e) hybridizing individual third modified library molecules of step (d) with a fourth tailed primer and conducting a fourth primer extension reaction (or fourth PCR), wherein the fourth tailed primers comprise a 5' region carrying a fourth right universal adaptor sequence and a 3' region that is complementary to at least a portion of the third right universal adaptor sequence of the third modified library molecule, wherein the fourth primer extension reaction (or fourth PCR) generates a plurality of fourth modified library molecules each appended at one end with the fourth right universal adaptor sequence, wherein the fourth right universal adaptor sequence of the fourth tailed primers comprise
  - a universal binding sequence for a forward or reverse sequencing primer,
  - a universal binding sequence for a first or second surface primer,
  - a universal binding sequence for a forward or reverse amplification primer and/or
  - a universal binding sequence for a compaction oligonucleotide.

126. The method of claim 125, further comprising:

- f) hybridizing individual fourth modified library molecules of step (e) with a fifth tailed primer and conducting a fifth primer extension reaction (or fifth PCR), wherein the fifth tailed primers comprise a 5' region carrying a fifth left universal adaptor sequence and a 3' region that is complementary to at least a portion of the fourth left universal adaptor sequence of the fourth modified library molecule, wherein the fifth primer extension reaction (or fifth PCR) generates a plurality of fifth modified library molecules each appended at one end with the fifth left universal adaptor sequence, wherein the fifth left universal adaptor sequence of the fifth tailed primers comprise
  - a universal binding sequence for a forward or reverse sequencing primer,
  - a universal binding sequence for a first or second surface primer,
  - a universal binding sequence for a forward or reverse amplification primer and/or
  - a universal binding sequence for a compaction oligonucleotide; and
- g) hybridizing individual fifth modified library molecules of step (f) with a sixth tailed primer and conducting a sixth primer extension reaction (or sixth PCR), wherein the sixth tailed primers comprise a 5' region carrying a sixth right universal adaptor sequence and a 3' region that is complementary to at least a portion of the fourth right universal adaptor sequence of the fifth modified library molecule, wherein the sixth primer extension reaction (or sixth PCR) generates a plurality of sixth modified library molecules each appended at one end with the sixth right universal adaptor sequence, wherein the sixth right universal adaptor sequence of the sixth tailed primers comprise
  - a universal binding sequence for a forward or reverse sequencing primer,
  - a universal binding sequence for a first or second surface primer,
  - a universal binding sequence for a forward or reverse amplification primer and/or
  - a universal binding sequence for a compaction oligonucleotide.

127. The method of any one of claims 123-126, wherein the first tailed primer, the third tailed primer and/or the fifth tailed primer further comprises a left index sequence.

128. The method of any one of claims 123-126, wherein the first tailed primer, the third tailed primer and/or the fifth tailed primer further comprises a left unique identification sequence.
129. The method of any one of claims 123-126, wherein the second tailed primer, the fourth tailed primer and/or the sixth tailed primer further comprises a right index sequence.
130. The method of any one of claims 123-126, wherein the second tailed primer, the fourth tailed primer and/or the sixth tailed primer further comprises a right unique identification sequence.
131. The method of claim 123, wherein the appended third left universal adaptor sequence of step (b) can bind a first region (320) of a first splint strand.
132. The method of claim 123, wherein the appended third right universal adaptor sequence of step (c) can bind a second region (330) of a first splint strand.
133. The method of claim 125, wherein the appended fourth left universal adaptor sequence of step (d) can bind a first region (320) of a first splint strand.
134. The method of claim 125, wherein the appended fourth right universal adaptor sequence of step (e) can bind a second region (330) of a first splint strand.
135. The method of claim 126, wherein the appended fifth left universal adaptor sequence of step (f) can bind a first region (320) of a first splint strand.
136. The method of claim 126, wherein the appended fifth right universal adaptor sequence of step (g) can bind a second region (330) of a first splint strand.
137. A method for forming a plurality of library-splint complexes (500) comprising:
  - a) providing a plurality of double-stranded splint adaptors (200) wherein individual double-stranded splint adaptors (200) comprise a first splint strand (300) hybridized to a second splint strand (400), wherein the first splint strand (300) comprises regions arranged in a 5' to 3' order a first region (320), an internal

region (310), and a second region (330), and wherein the internal region of the first splint strand (310) is hybridized to the second splint strand (400), wherein the second splint strand comprises regions arranged in a 5' to 3' order **(i)** a second sub-region having a universal binding sequence for a first surface primer, and **(ii)** a first sub-region having a universal binding sequence for a second surface primer; and

- b) hybridizing the plurality of double-stranded splint adaptors with a plurality of single-stranded nucleic acid library molecules (100) wherein individual library molecules comprise regions arranged in a 5' to 3' order: **(i)** a first left universal adaptor sequence (120) having a binding sequence for a third surface primer; **(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 fourth surface primer, wherein the hybridizing is conducted under a condition suitable to hybridize portions of the first splint strand (300) to portions of the library molecule (100) thereby circularizing the library molecule to generate a library-splint complex (500), such that the first region (320) of the first splint strand is hybridized to the binding sequence for the third surface primer (120), and the third region (330) of the first splint strand is hybridized to the binding sequence for the fourth surface primer (130), wherein the library-splint complex (500) comprises a first nick between the 5' end of the library molecule and the 3' end of the second splint strand (400), wherein the library-splint complex (500) comprises a second nick between the 5' end of the second splint strand (400) and the 3' end of the library molecule (100), and wherein the first and second nicks are enzymatically ligatable.

138. The method of claim 137, wherein the plurality of single-stranded nucleic acid library molecules (100) further comprise a first left index sequence (160) and/or a first right index sequence (170).

139. The method of claim 137, where 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).
140. The method of claim 137, further comprising: contacting the plurality of the double-stranded splint adaptors (200) and the plurality of the single-stranded nucleic acid library molecules (100) with a ligase, under a condition suitable to enzymatically ligate the first and second nicks, thereby generating a plurality of covalently closed circular library molecules (600) each hybridized to the first splint strand (300).
141. The method of claim 140, further comprising: contacting the plurality of covalently closed circular library molecules (600) with at least one exonuclease enzyme to remove the plurality of first splint strands (300) and retaining the plurality of covalently closed circular library molecules (600).
142. The method of claim 141, wherein individual covalently closed circular library molecules (600) in the plurality comprise a second splint strand region (400) which includes the universal binding sequence for the second surface primer, and wherein the method further comprises: distributing the retained plurality of covalently closed circular library molecules (600) onto a support having a plurality of the second surface primers immobilized on the support, under a condition suitable for hybridizing individual covalently closed circular library molecules (600) to individual immobilized second surface primers thereby immobilizing the plurality of covalently closed circular library molecules (600).
143. The method of claim 142, further comprising: contacting the plurality of immobilized covalently closed circular library molecules (600) with a plurality of strand-displacing polymerases and a plurality of nucleotides, under a condition suitable to conduct 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 (600) as template molecules, thereby generating a plurality of immobilized nucleic acid concatemer molecules.

144. The method of claim 143, wherein the plurality of nucleotides comprises dATP, dGTP, dCTP, dTTP and/or dUTP.
145. The method of claim 142, wherein the plurality of immobilized second surface capture primers on the support are located at pre-determined or random locations on the support.
146. The method of claim 142, wherein the plurality of immobilized second surface capture 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 essentially simultaneously reacted with the reagents in a massively parallel manner, optionally wherein the solution of reagents comprises enzymes, nucleotides and divalent cations.
147. The method of claim 143, wherein the density of the plurality of immobilized nucleic acid concatemer molecules on the support is  $10^4 - 10^8$  per  $\text{mm}^2$ .
148. The method of claim 142, further comprising: sequencing the plurality of immobilized concatemers, wherein the sequencing comprises:
- a) contacting the plurality of immobilized concatemer molecules with (i) a plurality of sequencing polymerases and (ii) a plurality of the soluble 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 concatemer molecule hybridized to a soluble 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 sequencing primers thereby generating a plurality of nascent extended sequencing primers; and

- d) detecting the incorporated nucleotide and identifying the nucleo-base of the incorporated nucleotide.
149. The method of claim 148, wherein the plurality of nucleotides comprise 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.
150. The method of claim 148, wherein the plurality of nucleotides comprise one type of nucleotide selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.
151. The method of claim 148, wherein the plurality of nucleotides comprise a mixture of any combination of two or more types of nucleotides selected from a group consisting of dATP, dGTP, dCTP, dTTP and/or dUTP.
152. The method of claim 142, further comprising: sequencing the plurality of immobilized concatemers, wherein the sequencing comprises:
- a) contacting the plurality of immobilized concatemer molecules with (i) a plurality of sequencing polymerases and (ii) a plurality of the soluble universal sequencing primers, wherein the contacting is conducted under a condition suitable to form a plurality of first complexed polymerases each 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 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; and

- c) detecting the plurality of multivalent-complexed polymerases.

153. The method of claim 152, further comprising:

- d) 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;
- e) contacting the plurality of the retained nucleic acid duplexes of step d) 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;
- f) 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, 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 e) 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.

154. The method of claim 153, further comprising:

- g) detecting the complementary nucleotides which are incorporated into the sequencing primers of the nucleotide-complexed polymerases.

155. The method of claim 153, further comprising:

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

156. A method for sequencing by forming at least one avidity complex, comprising:

- a) binding a first universal sequencing primer, a first sequencing polymerase, and a first detectably labeled multivalent molecule to a first portion of a concatemer molecule of claim 16, thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first sequencing polymerase;
- b) binding a second universal sequencing primer, a second sequencing polymerase, and the first detectably labeled multivalent molecule to a second portion of the same concatemer molecule thereby forming a second binding complex, wherein a second nucleotide unit of the first 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, wherein the first detectably labeled multivalent molecule comprises a core attached to multiple nucleotide arms and each nucleotide arm is attached to a nucleotide unit, wherein the concatemer molecule comprises two or more tandem repeat sequences of a sequence of interest (110) and a universal primer binding site that binds the first and second universal sequencing primers, and 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;
- c) detecting the first and second binding complexes on the same concatemer molecule, and
- d) identifying the first nucleotide unit in the first binding complex thereby determining the sequence of the first portion of the concatemer template molecule, and identifying the second nucleotide unit in the second binding complex thereby determining the sequence of the second portion of the concatemer template molecule.

157. The method of claim 152, wherein the plurality of nucleotide arms attached to the core of the individual multivalent molecules have the same type of a nucleotide unit, and wherein the types of nucleotide unit is selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.
158. The method of claim 152, wherein the plurality of multivalent molecules comprise a mixture of any combination of two or more types of multivalent molecules each type having nucleotide units selected from a group consisting of dATP, dGTP, dCTP, dTTP and/or dUTP.
159. The method of claim 153, wherein the plurality of nucleotides comprise 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.
160. The method of claim 153, wherein the plurality of nucleotides comprise one type of nucleotide selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.
161. The method of claim 153, wherein the plurality of nucleotides comprise a mixture of any combination of two or more types of nucleotides selected from a group consisting of dATP, dGTP, dCTP, dTTP and/or dUTP.
162. The method of claim 142, wherein the support comprises a glass or plastic substrate.
163. The method of claim 142, wherein the support is passivated with at least one hydrophilic polymer coating having a water contact angle of no more than 45 degrees.
164. The method of claim 142, wherein the at least one hydrophilic polymer coating comprises a molecule selected from a group consisting of polyethylene glycol (PEG), poly(vinyl alcohol) (PVA), poly(vinyl pyridine), poly(vinyl pyrrolidone) (PVP),

poly(acrylic acid) (PAA), polyacrylamide, poly(N-isopropylacrylamide) (PNIPAM), poly(methyl methacrylate) (PMA), poly(2-hydroxyethyl methacrylate) (HEMA), poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), polyglutamic acid (PGA), poly-lysine, poly-glucoside, streptavidin, and dextran.

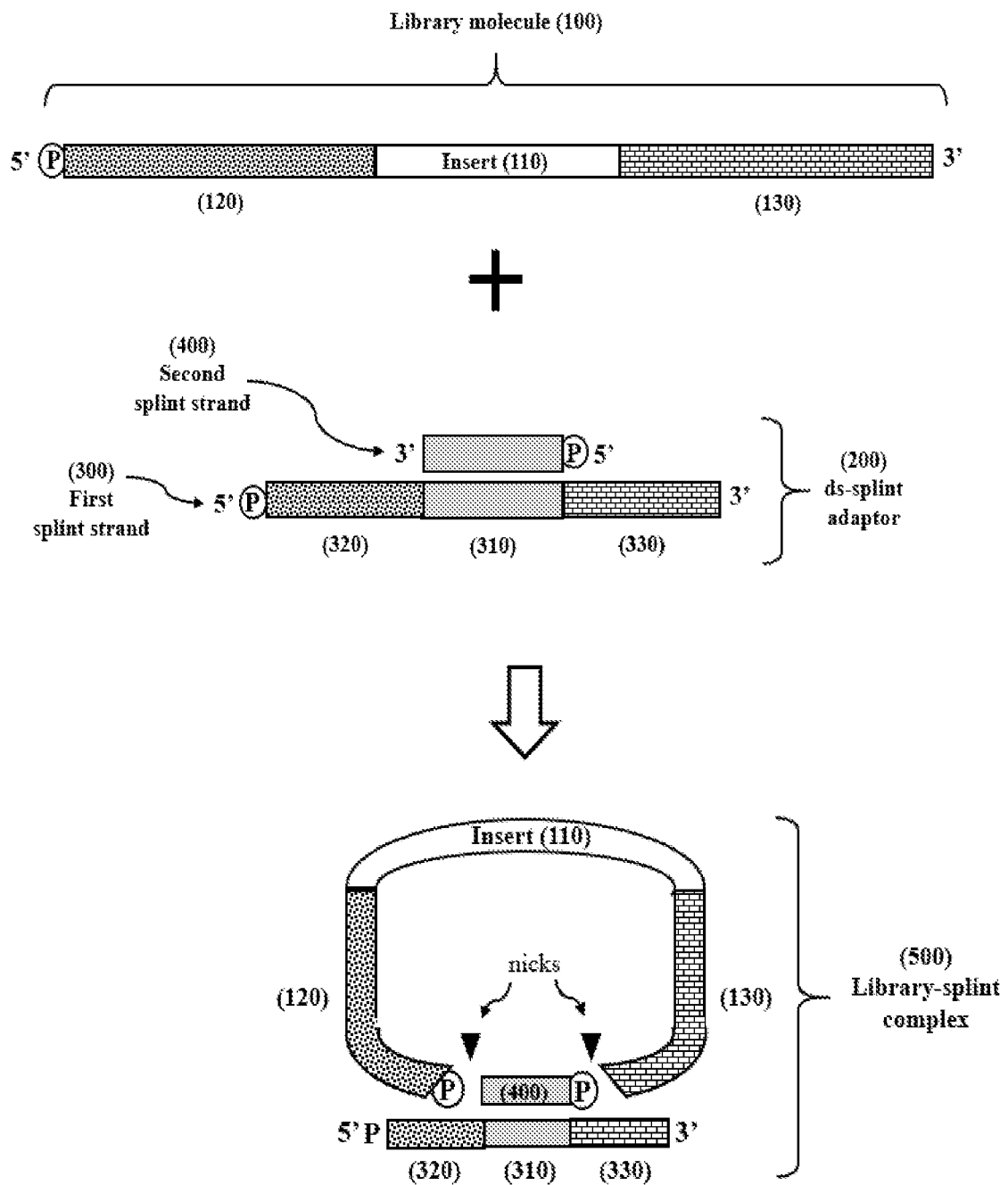


FIG. 1

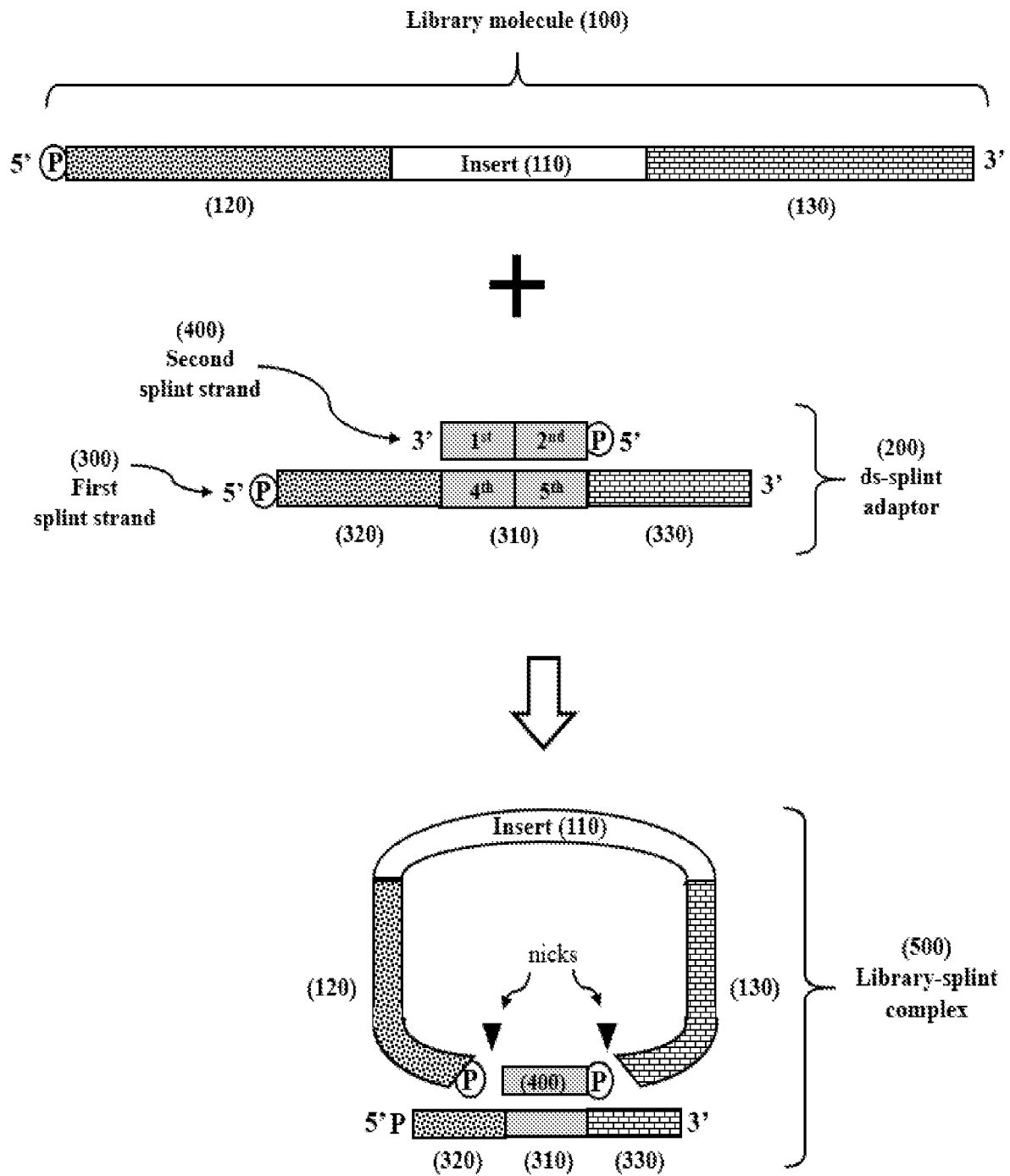


FIG. 2

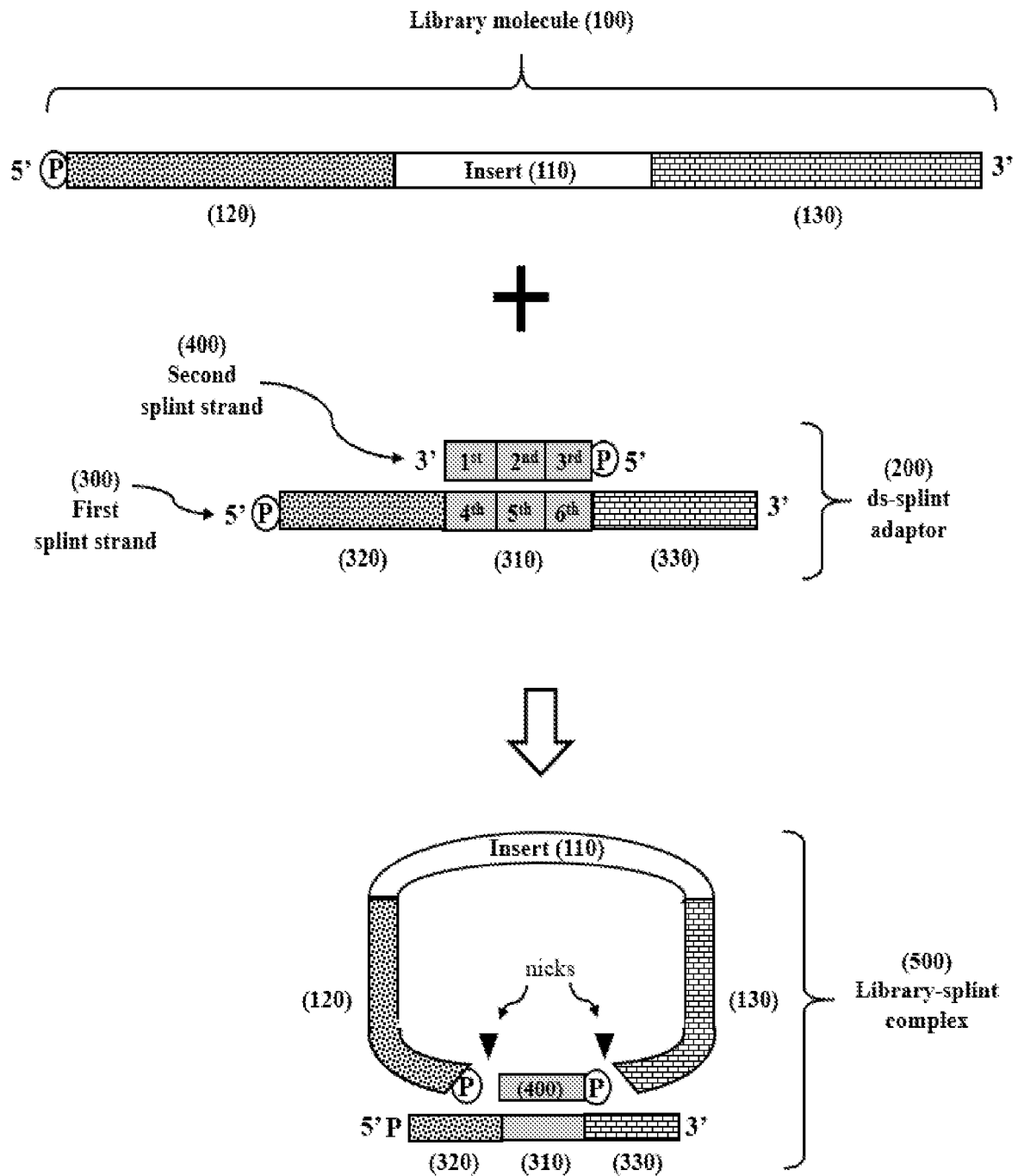


FIG. 3

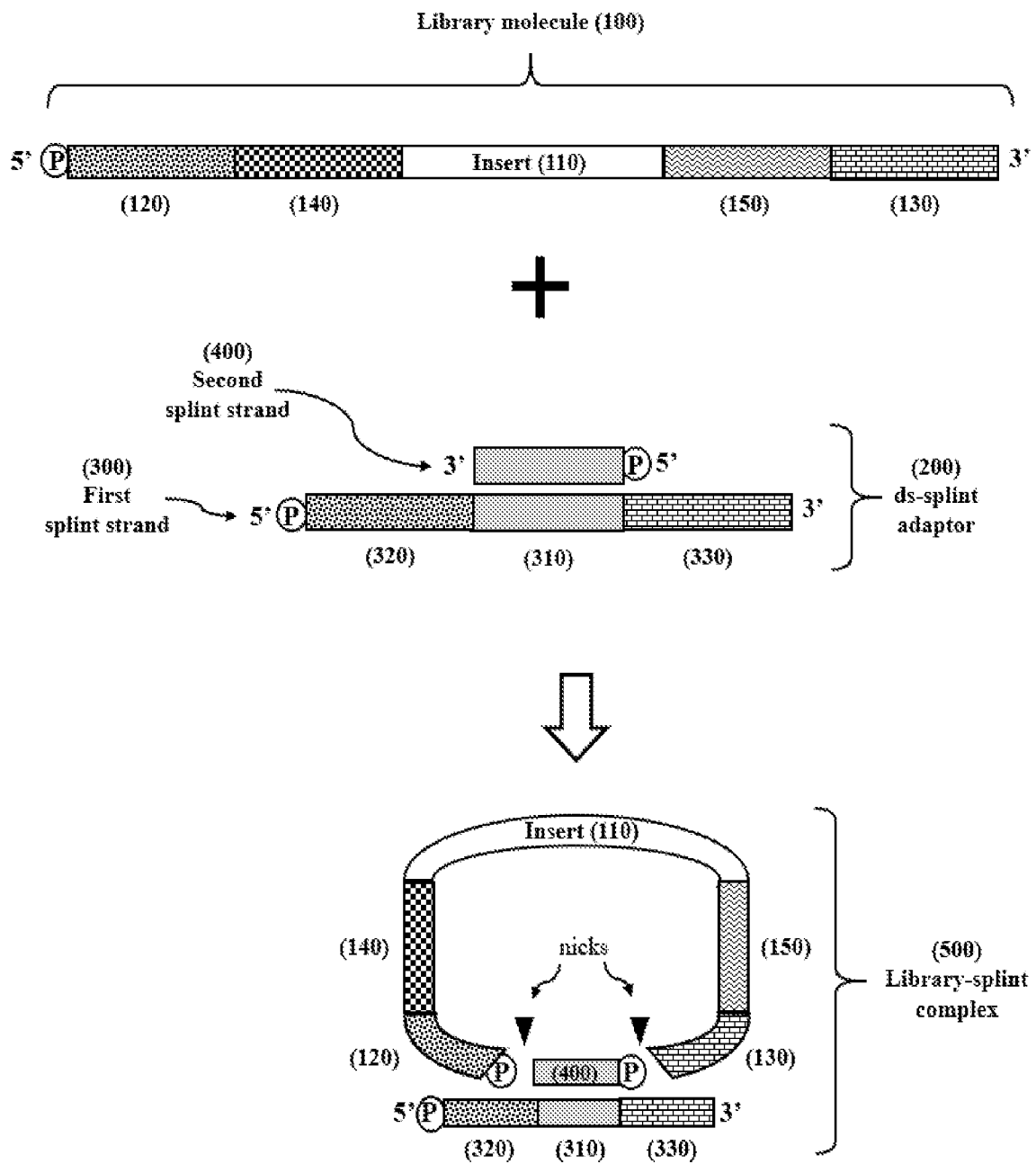


FIG. 4

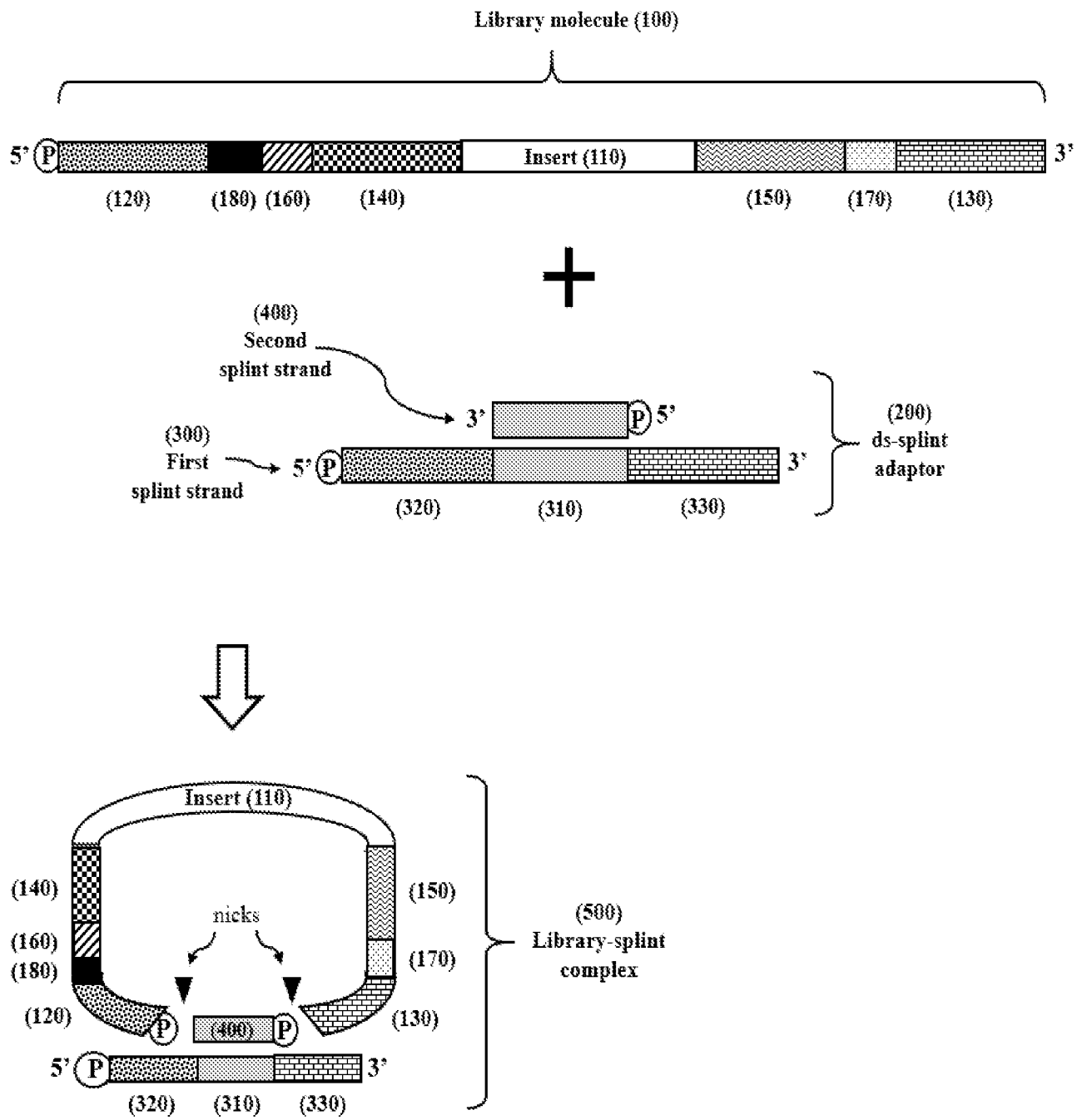


FIG. 5

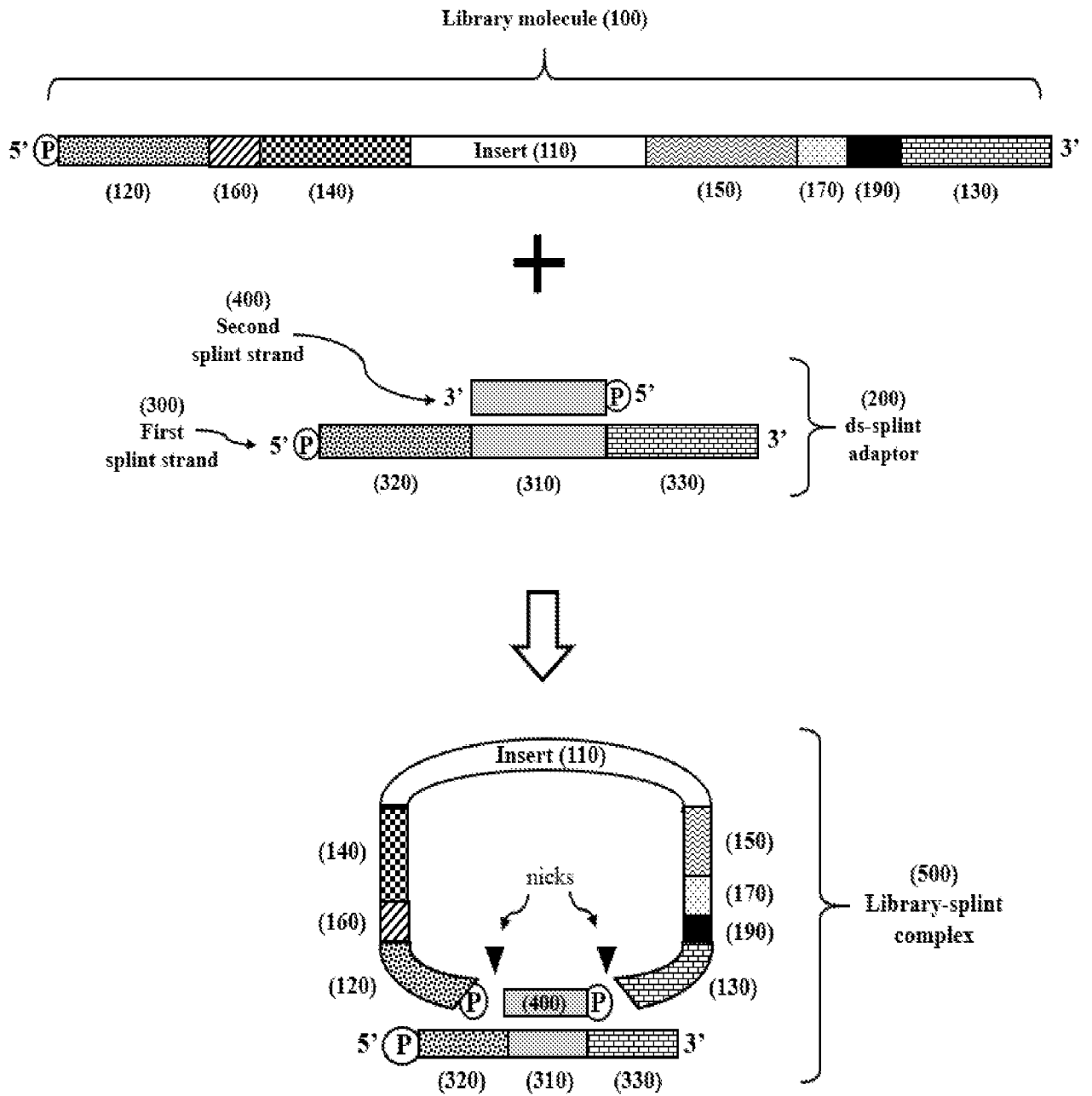


FIG. 6

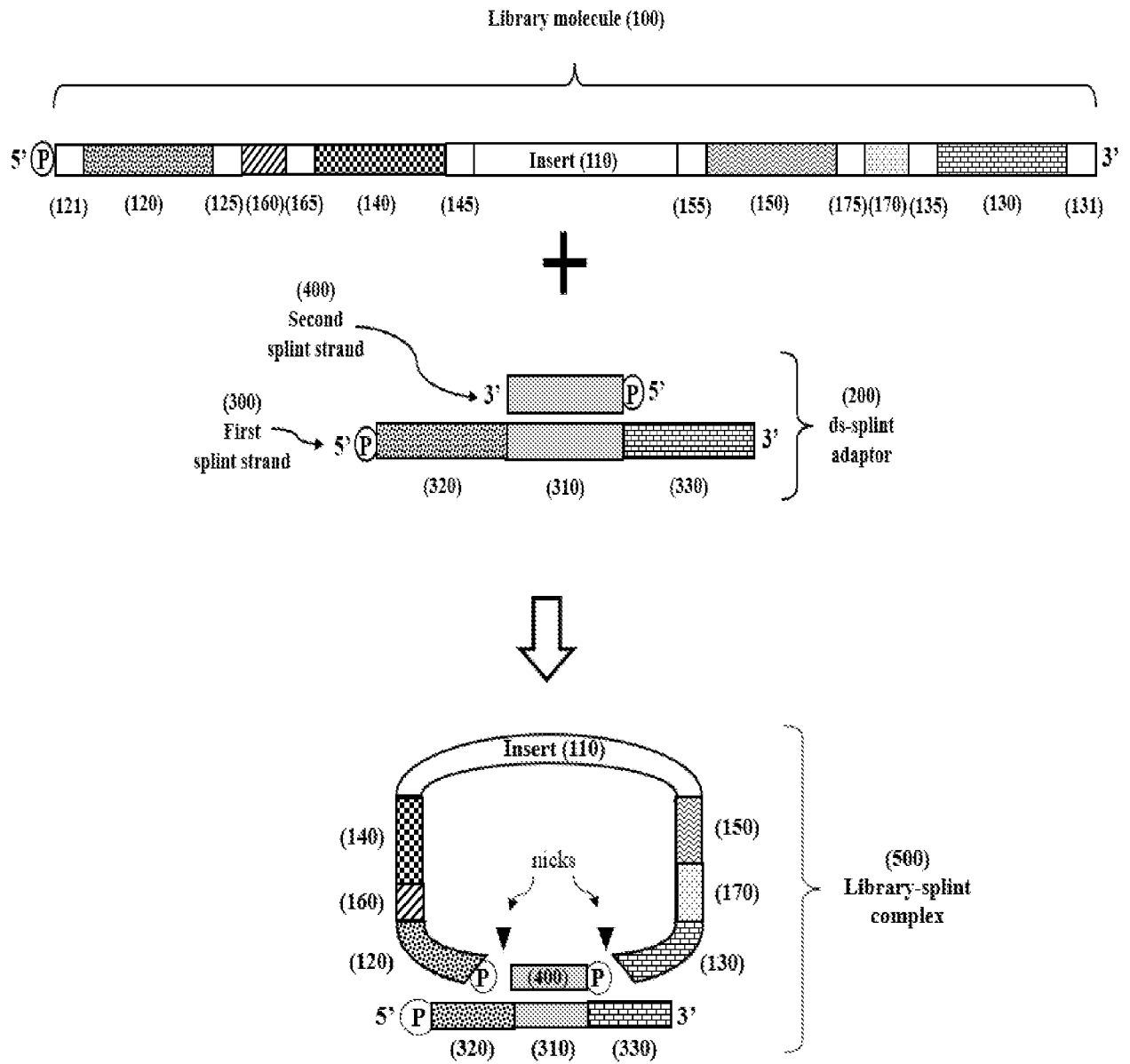


FIG. 7

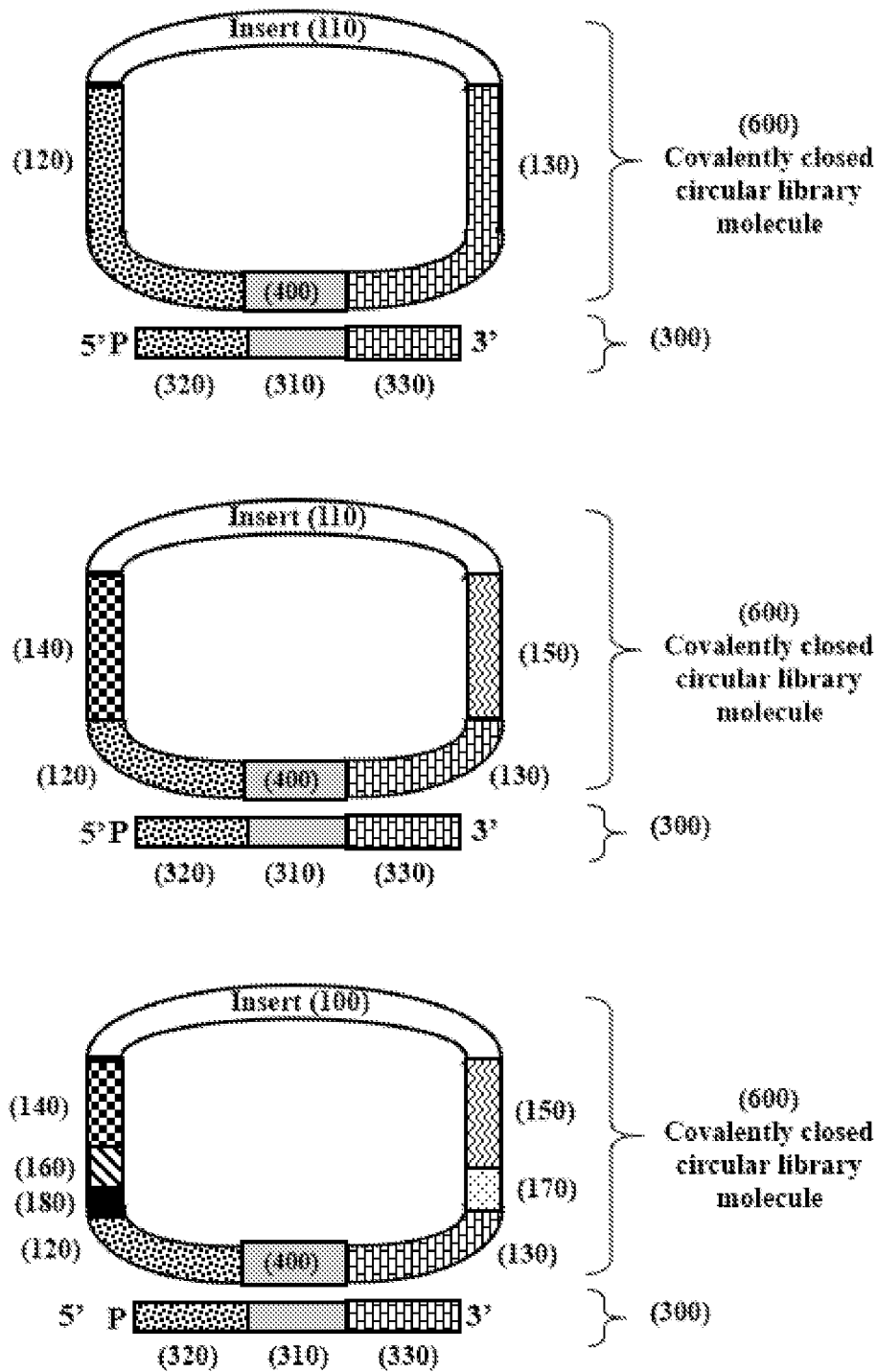


FIG. 8

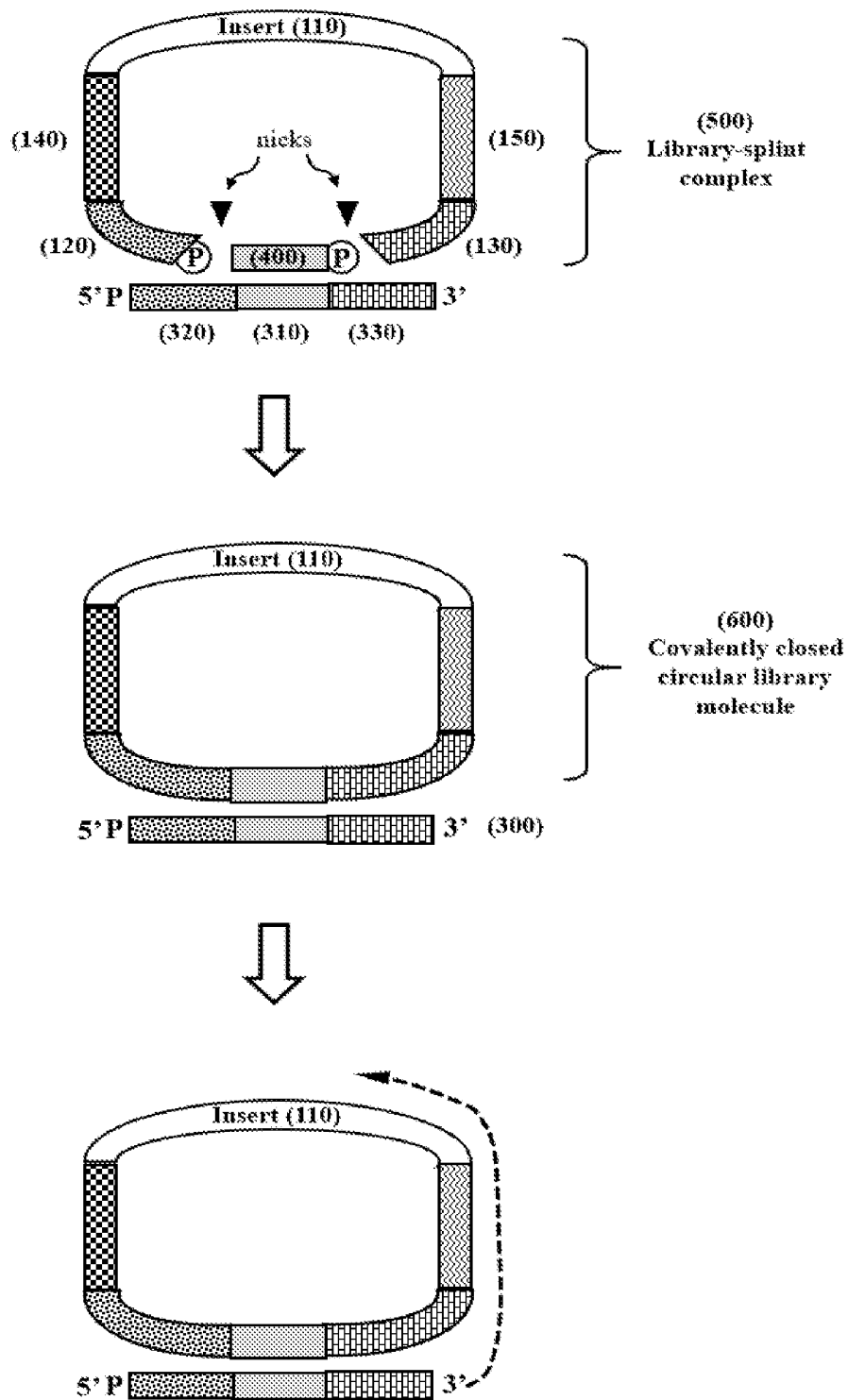


FIG. 9

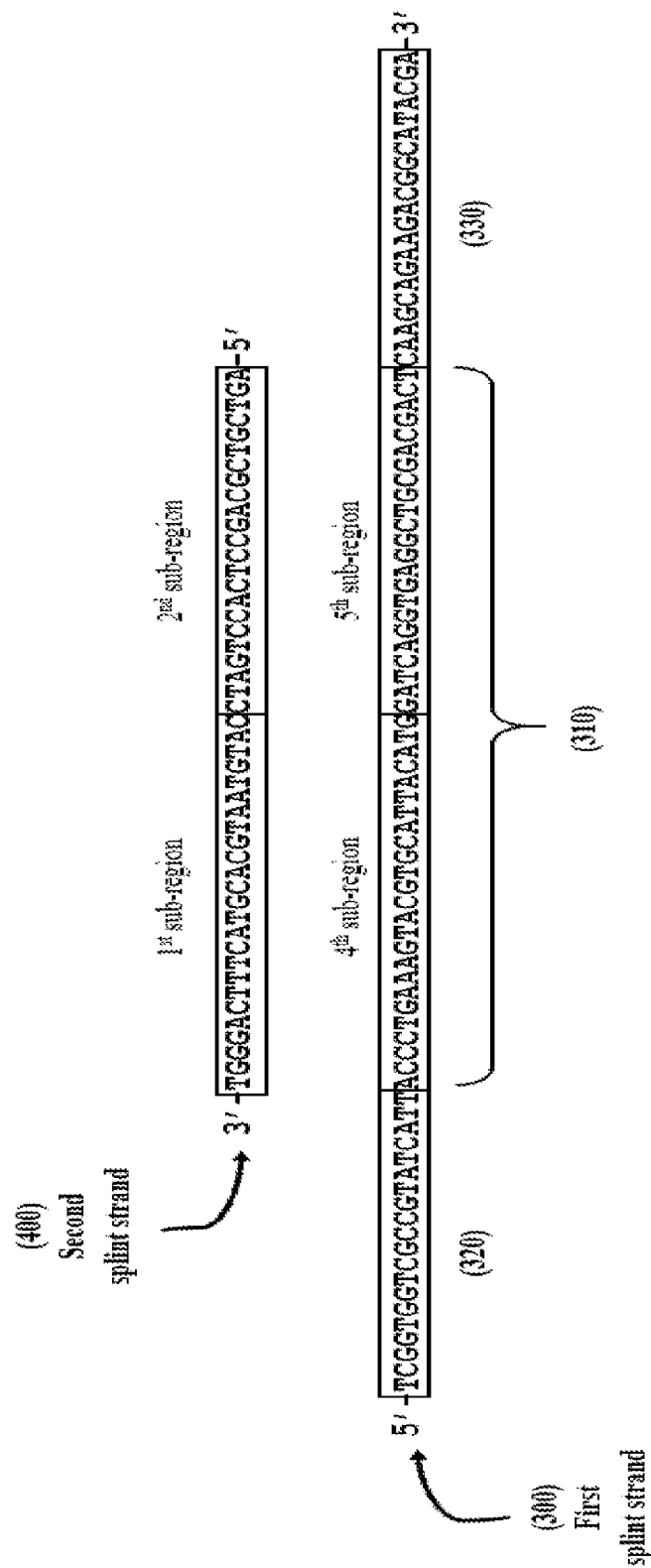


FIG. 10

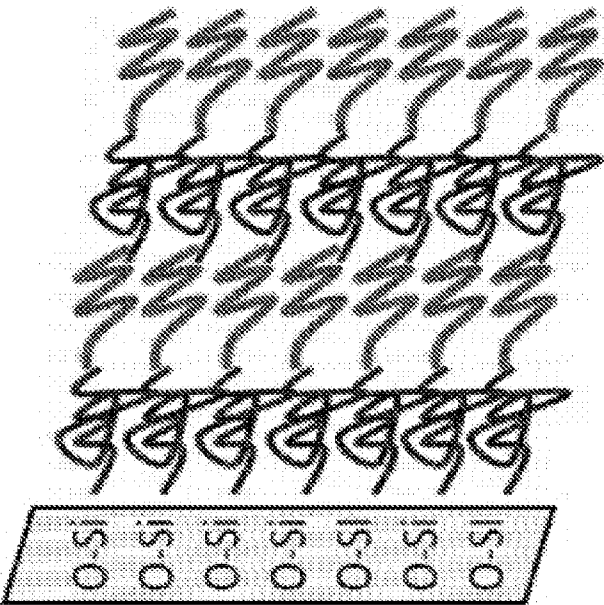


FIG. 11

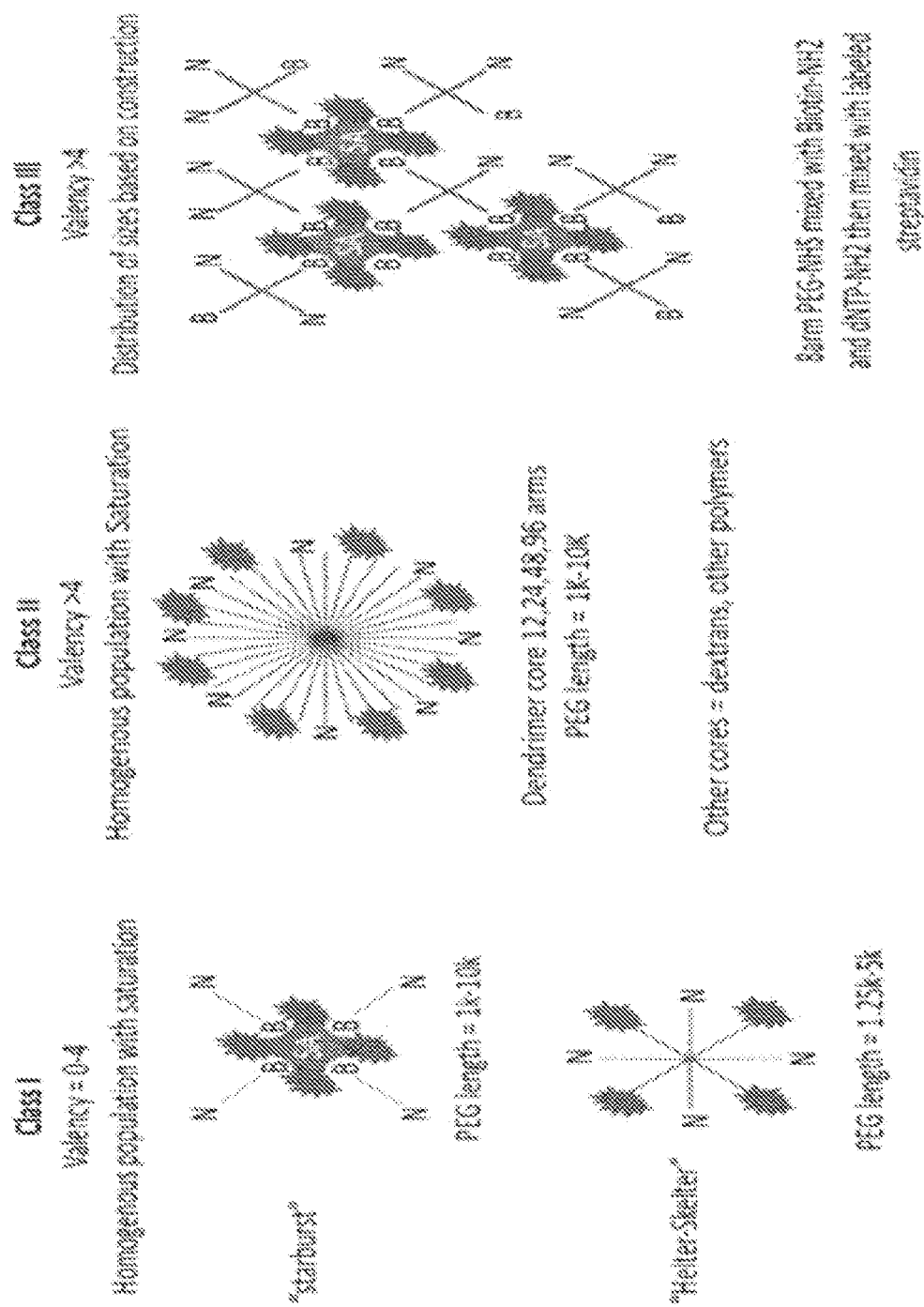


FIG. 12

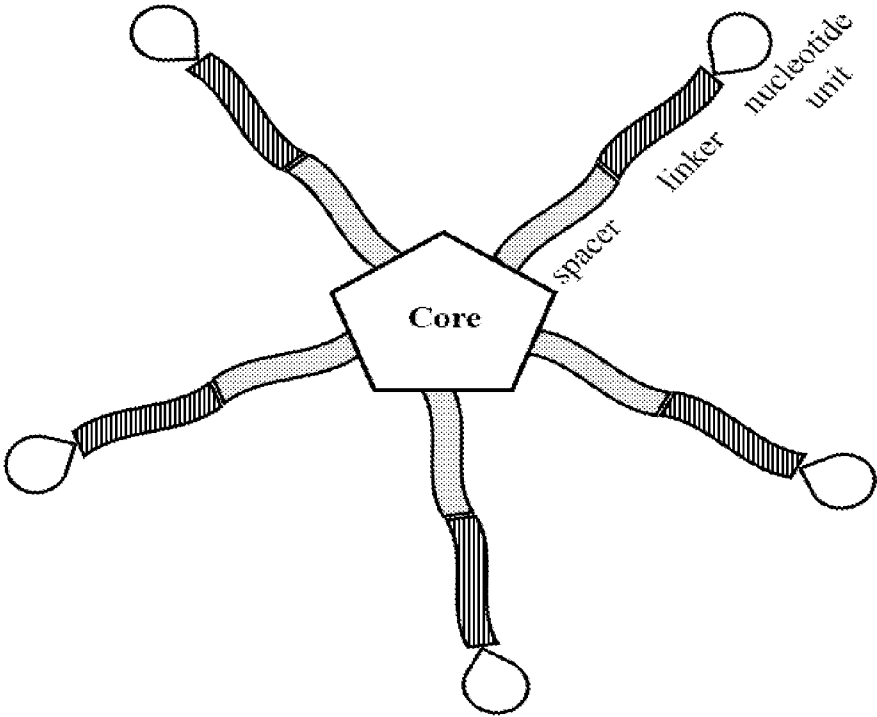


FIG. 13

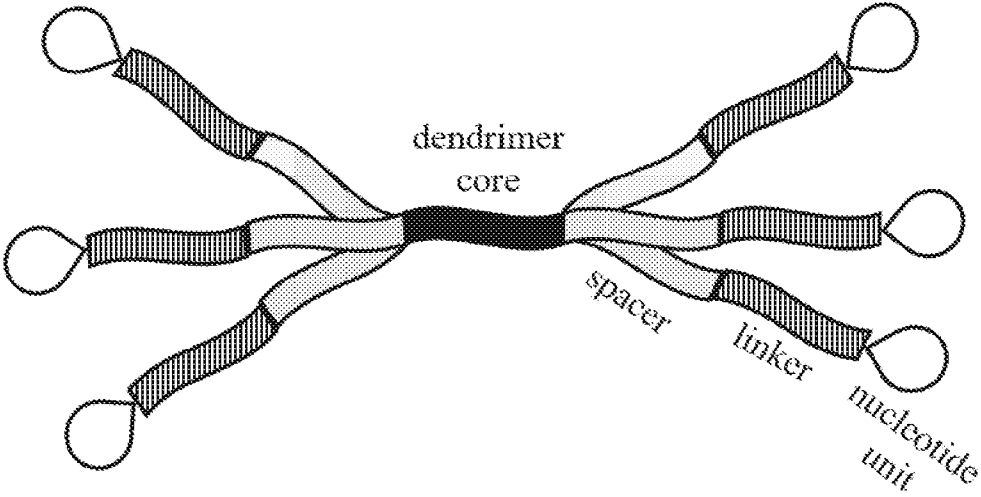
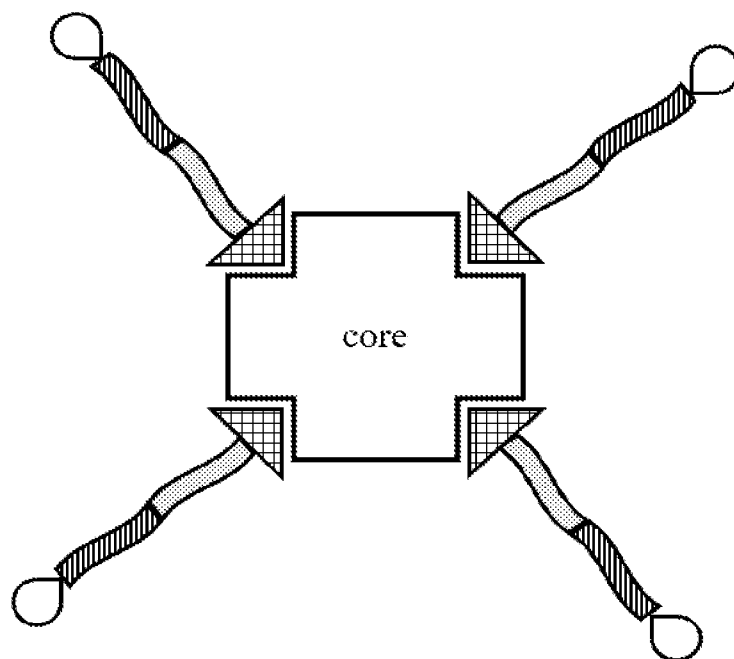
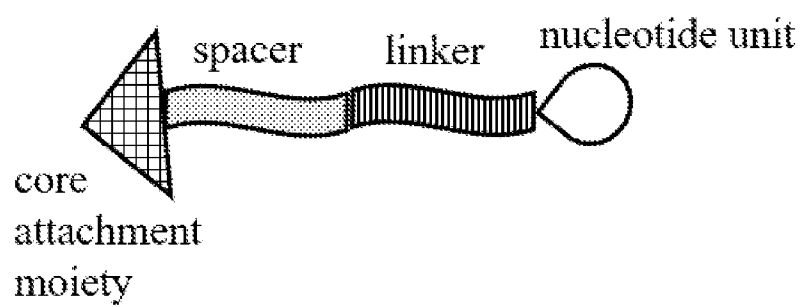
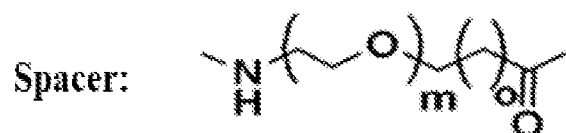


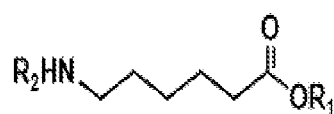
FIG. 14

**FIG. 15****FIG. 16**

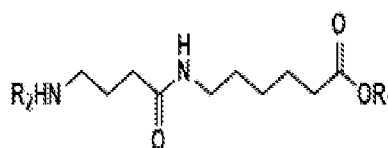


**Linkers:**

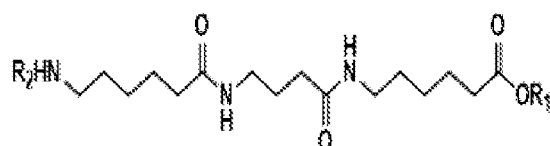
11 atom Linker:



16 atom Linker:



23 atom Linker:



N3 Linker:

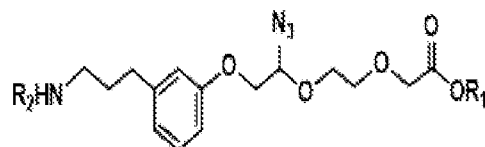


**FIG. 17**

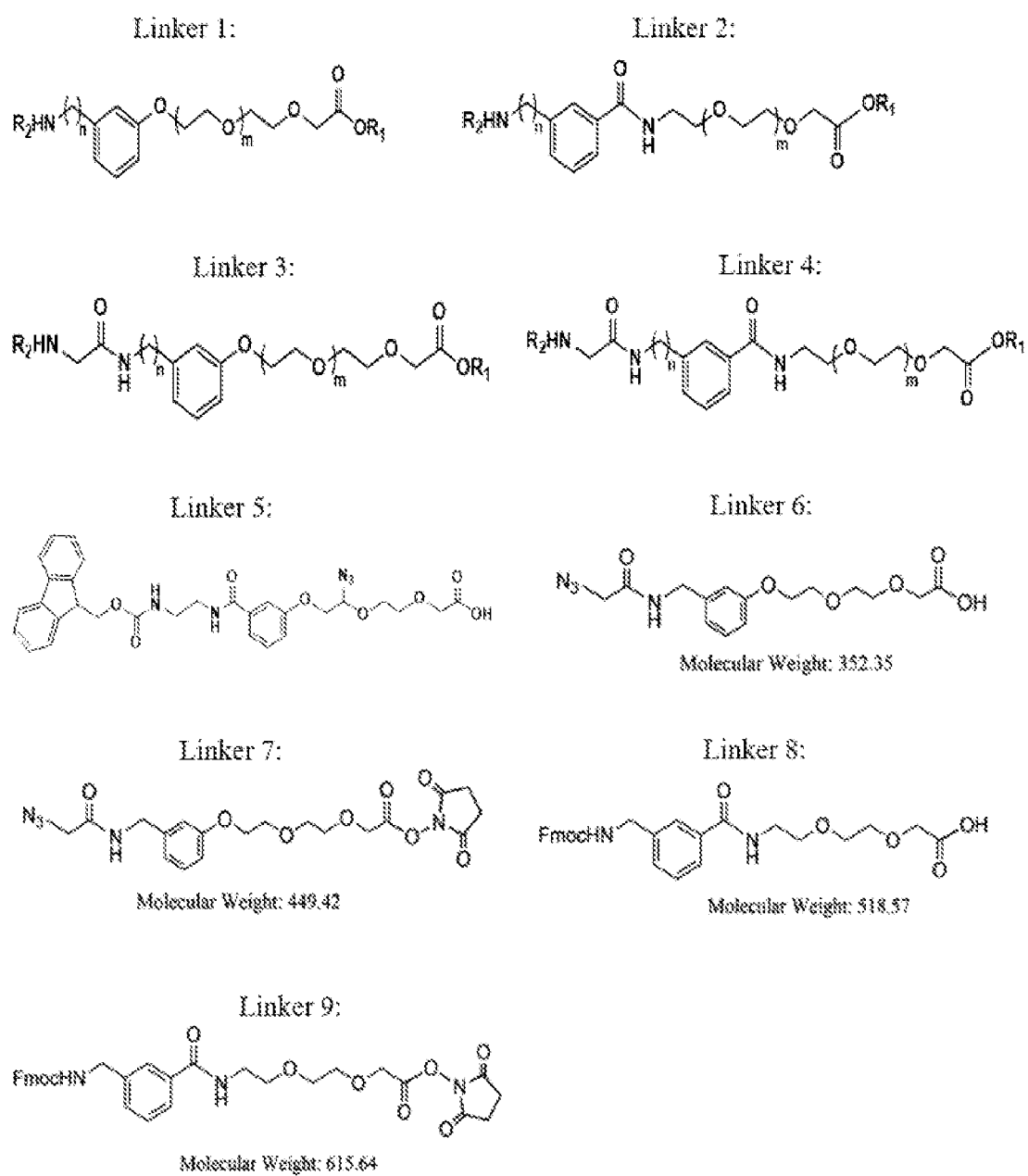
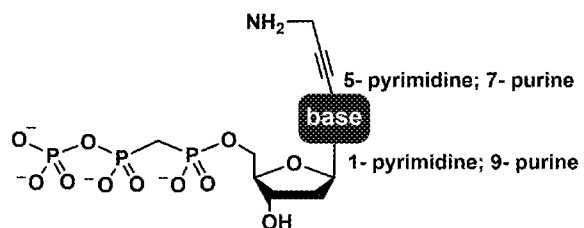
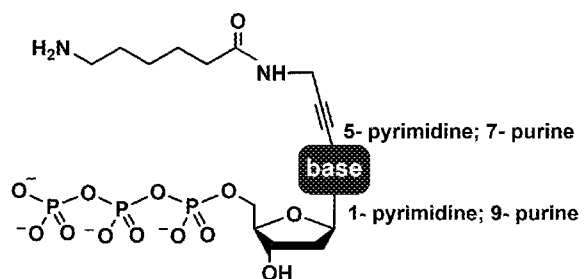
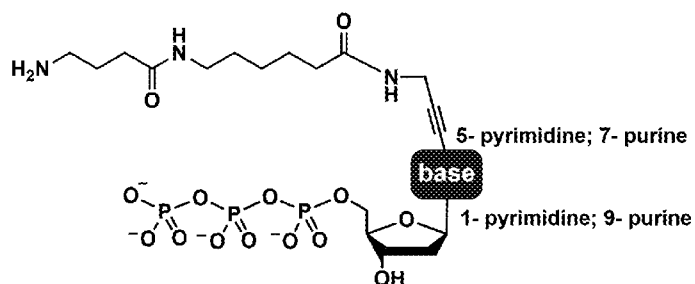
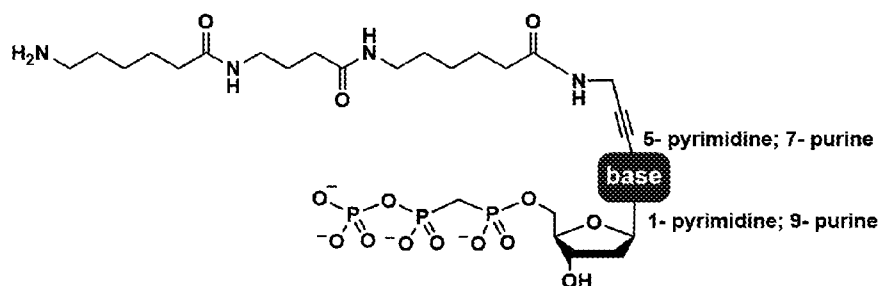
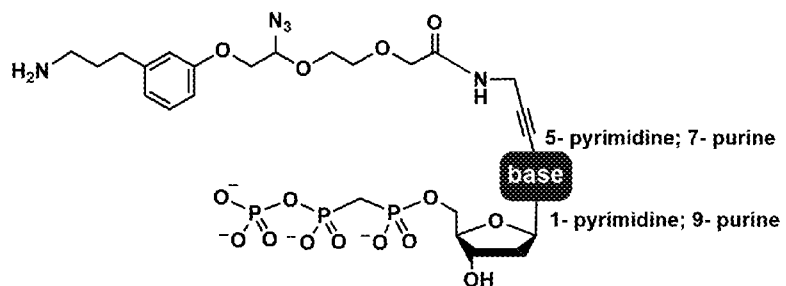
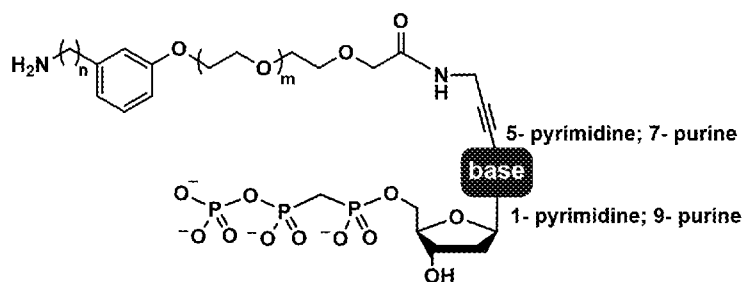
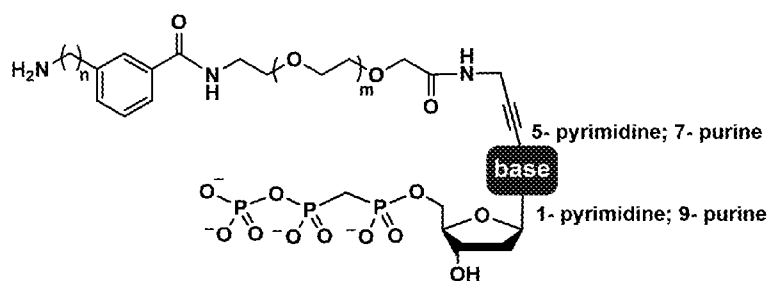
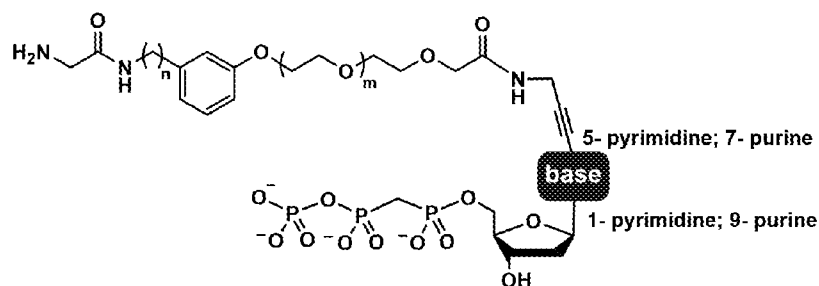
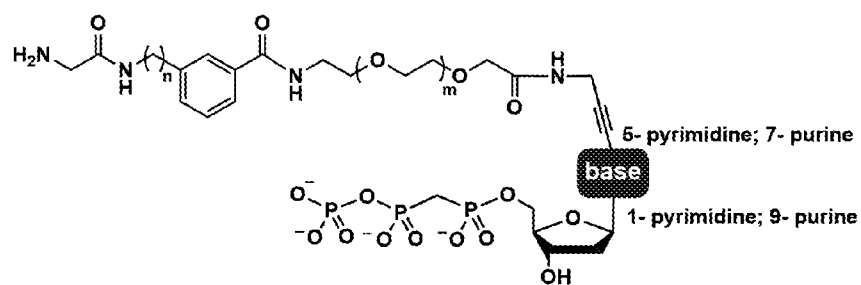
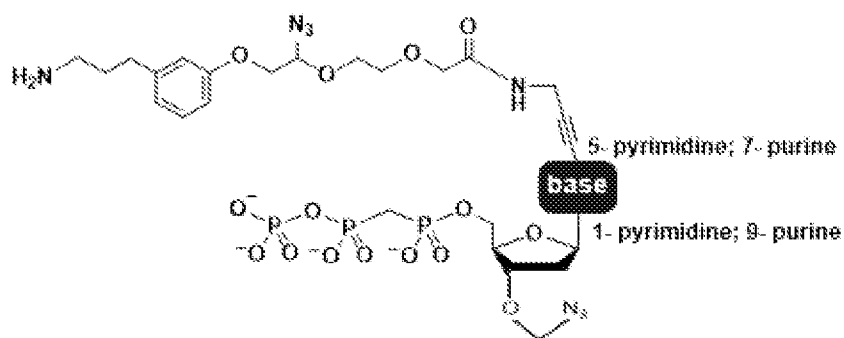


FIG. 18

**dNTP-PA-NH<sub>2</sub>:****dNTP-PA-11 Atom Linker-NH<sub>2</sub>:****dNTP-PA-16 Atom Linker-NH<sub>2</sub>:****dNTP-PA-23 Atom Linker-NH<sub>2</sub>:****FIG. 19**

**dNTP-PA-N3 Linker-NH<sub>2</sub>:****dNTP-PA-Linker 1-NH<sub>2</sub>:****dNTP-PA-Linker 2-NH<sub>2</sub>:****FIG. 20**

**dNTP-PA-Linker 3-NH<sub>2</sub>:****dNTP-PA-Linker 4-NH<sub>2</sub>:****dNTP-PA-N3 Linker-NH<sub>2</sub>:****FIG. 21**

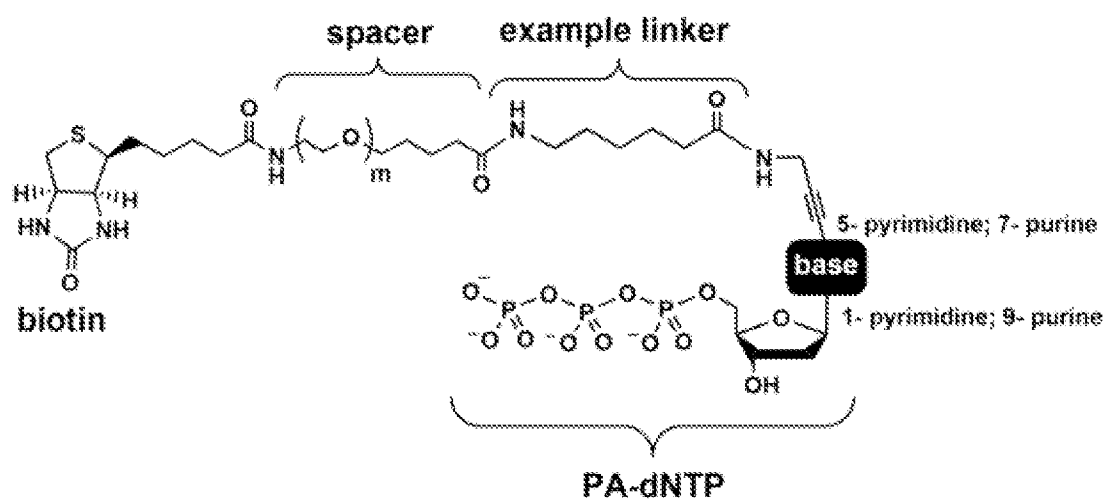


FIG. 22

**Table 1:**

| <b>First left index sequences (160)</b> |                                         |                   |
|-----------------------------------------|-----------------------------------------|-------------------|
| <b>Index Name</b>                       | <b>Sequence (orientation: 5' to 3')</b> | <b>SEQ ID NO:</b> |
| 007                                     | CCACTCATT                               | 1                 |
| 008                                     | AACTAACAC                               | 2                 |
| 037                                     | TTATGGAGA                               | 3                 |
| 022                                     | GAGTTCGCT                               | 4                 |
| 002                                     | TTGTTGTTC                               | 5                 |
| 035                                     | CCATATTCT                               | 6                 |
| 001                                     | AATCATGCG                               | 7                 |
| 014                                     | GGACAGCTC                               | 8                 |
| 004                                     | CCAACACGA                               | 9                 |
| 034                                     | TGTAAGCGG                               | 10                |
| 012                                     | ATAACTACC                               | 11                |
| 019                                     | CGCACTGTG                               | 12                |
| 020                                     | TTACACTCA                               | 13                |
| 045                                     | ATTGACCTG                               | 14                |
| 011                                     | CACCAGTGT                               | 15                |
| 038                                     | GGTGCTCCT                               | 16                |
| 033                                     | ATCCGCGTC                               | 17                |
| 026                                     | CGGTCCTAA                               | 18                |
| 046                                     | TGGTATGCC                               | 19                |
| 036                                     | AAGATAGAG                               | 20                |
| 015                                     | CCGGTATCG                               | 21                |
| 042                                     | GGTCCGAGA                               | 22                |
| 023                                     | CGTGATAGC                               | 23                |
| 081                                     | TATGTGGCC                               | 24                |
| 024                                     | GTACCAGAG                               | 25                |
| 016                                     | TACATCAGA                               | 26                |
| 028                                     | AGGCGACCT                               | 27                |
| 039                                     | CAGCACAAG                               | 28                |
| 031                                     | CTCTGTCCA                               | 29                |
| 013                                     | TCTTGTGAT                               | 30                |
| 043                                     | CCAGGAGTC                               | 31                |
| 025                                     | ACCGTTCGT                               | 32                |
| 044                                     | GACACTTAT                               | 33                |

**FIG. 23A**

**Table 1 (continued):**

| <b>First left index sequences (160)</b> |                                         |                   |
|-----------------------------------------|-----------------------------------------|-------------------|
| <b>Index Name</b>                       | <b>Sequence (orientation: 5' to 3')</b> | <b>SEQ ID NO:</b> |
| 005                                     | TTGGCTGGA                               | 34                |
| 017                                     | ATTGCGTAT                               | 35                |
| 080                                     | CTCGAAGAT                               | 36                |
| 094                                     | TGAACGGAT                               | 37                |
| 049                                     | AATACCGGT                               | 38                |
| 058                                     | GGCCTTCAA                               | 39                |
| 067                                     | TGAGAACAA                               | 40                |
| 079                                     | AGATCTCTA                               | 41                |
| 054                                     | GGCAACGCA                               | 42                |
| 086                                     | CGAATGACA                               | 43                |
| 051                                     | CAAGTGCAG                               | 44                |
| 059                                     | CAAGCAACT                               | 45                |
| 065                                     | TTCAGGTCT                               | 46                |
| 064                                     | CAGTAGGTA                               | 47                |
| 060                                     | ACGAAGGCT                               | 48                |
| 053                                     | TTCTATTGG                               | 49                |
| 062                                     | GTGTCTCAC                               | 50                |
| 077                                     | GCTGAATGT                               | 51                |
| 071                                     | GAAGATGTT                               | 52                |
| 088                                     | ATTCGTTGA                               | 53                |
| 095                                     | TCGCATAGT                               | 54                |
| 057                                     | TCATGCTTG                               | 55                |
| 050                                     | TGGAGTATA                               | 56                |
| 055                                     | AAGGTGATT                               | 57                |
| 063                                     | AGAGGAAGG                               | 58                |
| 069                                     | GATCGCGAA                               | 59                |
| 056                                     | CTTCGACAC                               | 60                |
| 066                                     | GCTATTATG                               | 61                |
| 082                                     | GA CTGATTA                              | 62                |
| 085                                     | ACGGCCAAC                               | 63                |
| 075                                     | CTTATCGCG                               | 64                |
| 061                                     | TCTCTCTGC                               | 65                |
| 073                                     | CAGCGTTCC                               | 66                |

**FIG. 23B**

**Table 1 (continued):**

| <b>First left index sequences (160)</b> |                                         |                   |
|-----------------------------------------|-----------------------------------------|-------------------|
| <b>Index Name</b>                       | <b>Sequence (orientation: 5' to 3')</b> | <b>SEQ ID NO:</b> |
| 092                                     | GATTCACGG                               | 67                |
| 074                                     | TCCTCGCAA                               | 68                |
| 072                                     | ACCACAATT                               | 69                |
| 097                                     | CGTTGAATT                               | 70                |
| 106                                     | CAGTTGACC                               | 71                |
| 111                                     | CCACGAACG                               | 72                |
| 112                                     | AGCACCTAG                               | 73                |
| 113                                     | TAGGCCGTC                               | 74                |
| 114                                     | GTTCTGTAA                               | 75                |
| 115                                     | CACTGACGT                               | 76                |
| 116                                     | TCCAATACC                               | 77                |
| 117                                     | ATATTGGTG                               | 78                |
| 119                                     | CCGACCACT                               | 79                |
| 120                                     | GAACAACGA                               | 80                |
| 121                                     | ATTGTAACC                               | 81                |
| 123                                     | TCCACCTGT                               | 82                |
| 124                                     | CTACATGAA                               | 83                |
| 127                                     | CCAATGTTG                               | 84                |
| 132                                     | GTTACCAAC                               | 85                |
| 133                                     | CAATTGTGA                               | 86                |
| 134                                     | AGGCGTAAG                               | 87                |
| 135                                     | ACAGCCGTT                               | 88                |
| 136                                     | CACCAACCG                               | 89                |
| 137                                     | TCTAGCCTC                               | 90                |
| 138                                     | GTGTTAGGA                               | 91                |
| 139                                     | CGCGAGACT                               | 92                |
| 140                                     | TCAACTTAG                               | 93                |
| 141                                     | AAGAGCGCC                               | 94                |
| 143                                     | CGACCTTGA                               | 95                |
| 144                                     | TACGTACTC                               | 96                |

**FIG. 23C**

**Table 1 (continued):**

| <b>First right index sequences (170)</b> |                                         |                   |
|------------------------------------------|-----------------------------------------|-------------------|
| <b>Index Name</b>                        | <b>Sequence (orientation: 5' to 3')</b> | <b>SEQ ID NO:</b> |
| 001                                      | NNNGTAGGAGCC                            | 97                |
| 003                                      | NNNCCGCTGCTA                            | 98                |
| 004                                      | NNNAACAACAAG                            | 99                |
| 006                                      | NNNGGTGGTCTA                            | 100               |
| 008                                      | NNNTTGGCCAAC                            | 101               |
| 011                                      | NNNTCCATCGTT                            | 102               |
| 013                                      | NNNATTCTAGAG                            | 103               |
| 014                                      | NNNGGATGCAAT                            | 104               |
| 015                                      | NNNCAGGAGTGC                            | 105               |
| 016                                      | NNNATCACACTA                            | 106               |
| 018                                      | NNNGCGGATGAT                            | 107               |
| 019                                      | NNNCGAATCTGG                            | 108               |
| 021                                      | NNNATGCGTCCT                            | 109               |
| 022                                      | NNNGGTCTGAGC                            | 110               |
| 023                                      | NNNGCCTCATAG                            | 111               |
| 024                                      | NNNCTATACCTC                            | 112               |
| 025                                      | NNNAAGATTGGA                            | 113               |
| 026                                      | NNNTGCCAGCAT                            | 114               |
| 027                                      | NNNACTAGCTCA                            | 115               |
| 028                                      | NNNTAAGTACTG                            | 116               |
| 029                                      | NNNCGTGCAACG                            | 117               |
| 030                                      | NNNGTCCACGGA                            | 118               |
| 034                                      | NNNAGGTCCGTG                            | 119               |
| 035                                      | NNNCTCGTTAGT                            | 120               |
| 036                                      | NNNACACAATCC                            | 121               |
| 037                                      | NNNTCTTCGCAA                            | 122               |
| 038                                      | NNNGGTCACTTG                            | 123               |
| 039                                      | NNNCCGATATAT                            | 124               |
| 040                                      | NNNACAGGTCGC                            | 125               |
| 042                                      | NNNGTGCGGTTC                            | 126               |
| 043                                      | NNNCACGCACAT                            | 127               |
| 044                                      | NNNTGGATCACA                            | 128               |
| 045                                      | NNNATATTCGGT                            | 129               |

**FIG. 23D**

**Table 1 (continued):**

| <b>First right index sequences (170)</b> |                                         |                   |
|------------------------------------------|-----------------------------------------|-------------------|
| <b>Index Name</b>                        | <b>Sequence (orientation: 5' to 3')</b> | <b>SEQ ID NO:</b> |
| 047                                      | NNNCCAGAGATG                            | 130               |
| 048                                      | NNNTACCGTTGA                            | 131               |
| 049                                      | NNNATTGATACC                            | 132               |
| 051                                      | NNNCCAAGACCG                            | 133               |
| 052                                      | NNNAAGCCGGAC                            | 134               |
| 054                                      | NNNGGATCTCCG                            | 135               |
| 055                                      | NNNCCTGGAGAA                            | 136               |
| 056                                      | NNNATCCTCATC                            | 137               |
| 057                                      | NNNTATGTCTCT                            | 138               |
| 059                                      | NNNACGTAGAGT                            | 139               |
| 060                                      | NNNGTACTTCAA                            | 140               |
| 061                                      | NNNCATTCCAGC                            | 141               |
| 062                                      | NNNTGGCGTGAG                            | 142               |
| 063                                      | NNNTCAACGTCT                            | 143               |
| 064                                      | NNNAACGAAGTC                            | 144               |
| 065                                      | NNNCTTAATCGA                            | 145               |
| 066                                      | NNNGCGGCCTTA                            | 146               |
| 068                                      | NNNTTCCGAACG                            | 147               |
| 071                                      | NNNCGCACCGAA                            | 148               |
| 073                                      | NNNTAGTGACCA                            | 149               |
| 074                                      | NNNGTTATGCTG                            | 150               |
| 075                                      | NNNCTCCGCTAT                            | 151               |
| 076                                      | NNNTGAGATGGA                            | 152               |
| 078                                      | NNNGATAGATGT                            | 153               |
| 079                                      | NNNCCTCATGTC                            | 154               |
| 081                                      | NNNGACTTAACC                            | 155               |
| 082                                      | NNNCAAGGCGTT                            | 156               |
| 083                                      | NNNAGGAAGCCG                            | 157               |
| 084                                      | NNNTCACTCAAG                            | 158               |
| 085                                      | NNNTCCACTAGC                            | 159               |
| 087                                      | NNNCTGTCATGA                            | 160               |
| 088                                      | NNNAGCGTTCAG                            | 161               |
| 089                                      | NNNGCATAGGCA                            | 162               |

**FIG. 23E**

**Table 1 (continued):**

| <b>First right index sequences (170)</b> |                                         |                   |
|------------------------------------------|-----------------------------------------|-------------------|
| <b>Index Name</b>                        | <b>Sequence (orientation: 5' to 3')</b> | <b>SEQ ID NO:</b> |
| 091                                      | NNNTATCAACGC                            | 163               |
| 092                                      | NNNACAGTGTA                             | 164               |
| 096                                      | NNNTGCGGATAC                            | 165               |
| 097                                      | NNNGCTATTACT                            | 166               |
| 106                                      | NNNGCTCAATAA                            | 167               |
| 111                                      | NNNCAGAGCTCC                            | 168               |
| 112                                      | NNNACGGCACGA                            | 169               |
| 113                                      | NNNTTCCTTGGC                            | 170               |
| 114                                      | NNNGGTTGGATG                            | 171               |
| 115                                      | NNNCCACACCAT                            | 172               |
| 116                                      | NNNTACATATCG                            | 173               |
| 117                                      | NNNATTGCCATA                            | 174               |
| 119                                      | NNNCCATCTTGC                            | 175               |
| 120                                      | NNNTGGAATGCT                            | 176               |
| 121                                      | NNNAATTCGTCT                            | 177               |
| 123                                      | NNNCACCGACTC                            | 178               |
| 124                                      | NNNACAATCAGC                            | 179               |
| 127                                      | NNNCCGCGAACA                            | 180               |
| 132                                      | NNNGAGCATCTA                            | 181               |
| 133                                      | NNNCTATTAGAC                            | 182               |
| 134                                      | NNNTGTGGCAGT                            | 183               |
| 135                                      | NNNCAACCGCAG                            | 184               |
| 136                                      | NNNACCTATTCT                            | 185               |
| 137                                      | NNNTGTAGGCCA                            | 186               |
| 138                                      | NNNGTGGTTATC                            | 187               |
| 139                                      | NNNCACCAATGG                            | 188               |
| 140                                      | NNNACAACCGAT                            | 189               |
| 141                                      | NNNTGTCTGTAA                            | 190               |
| 143                                      | NNNTCCGCACTC                            | 191               |
| 144                                      | NNNGAGAGCAGA                            | 192               |

**FIG. 23F**

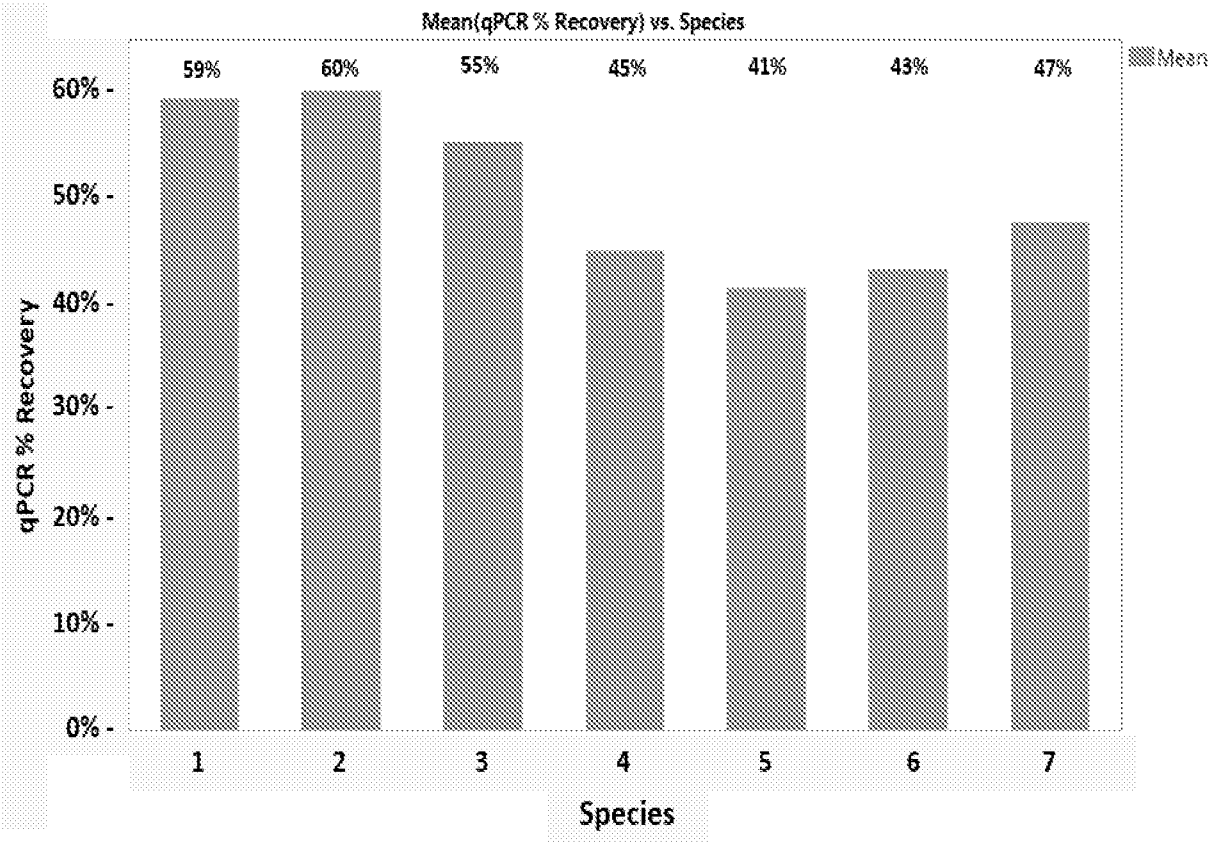


FIG. 24

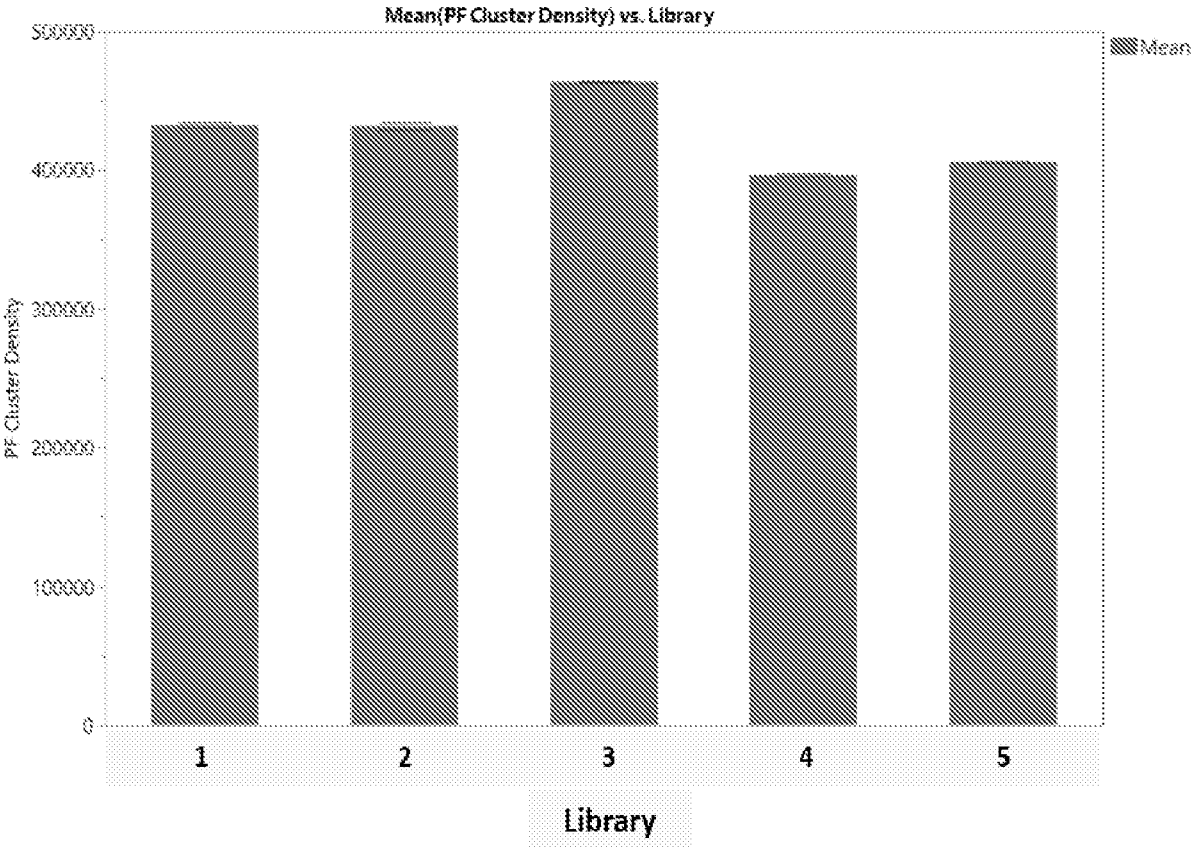


FIG. 25

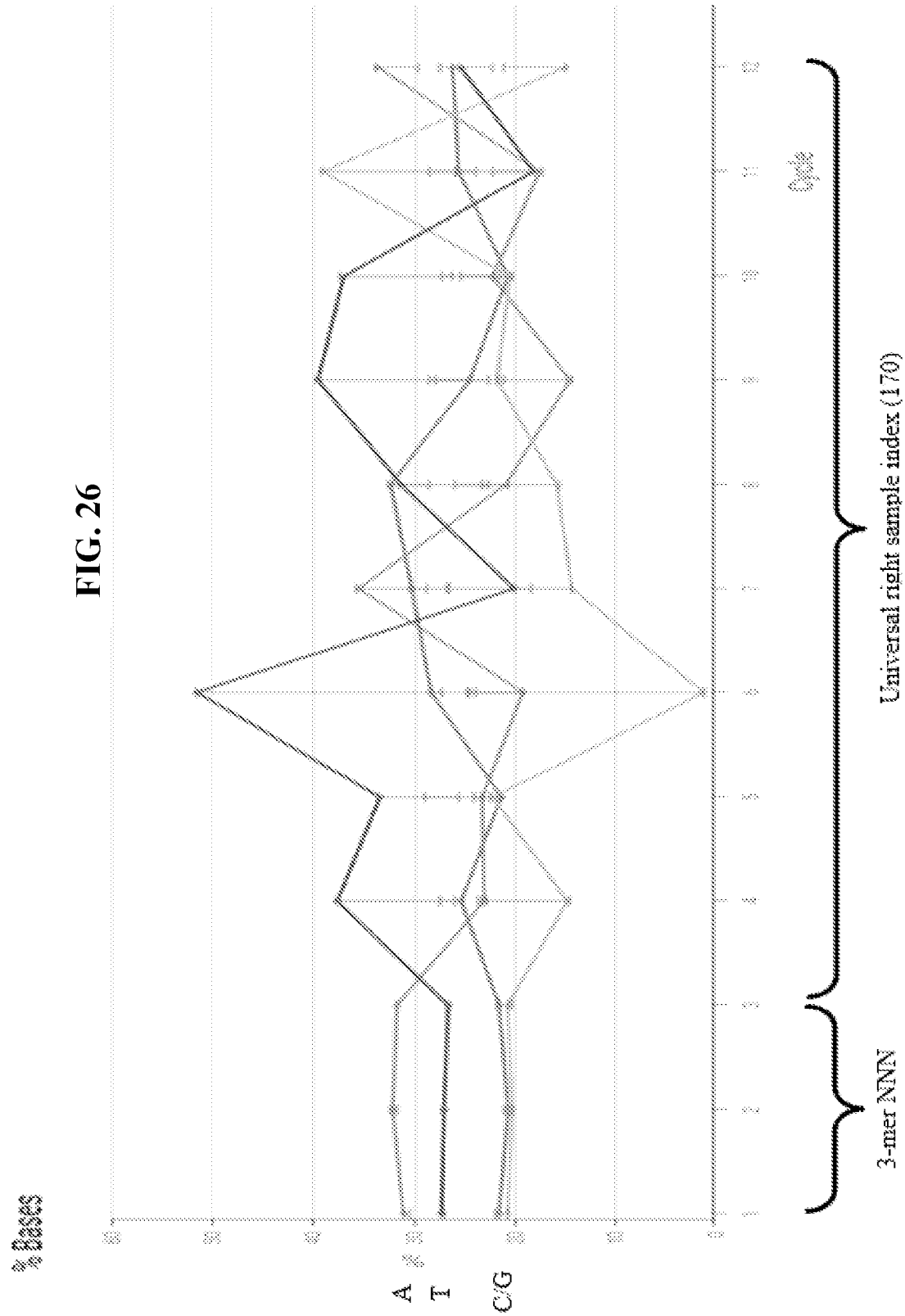
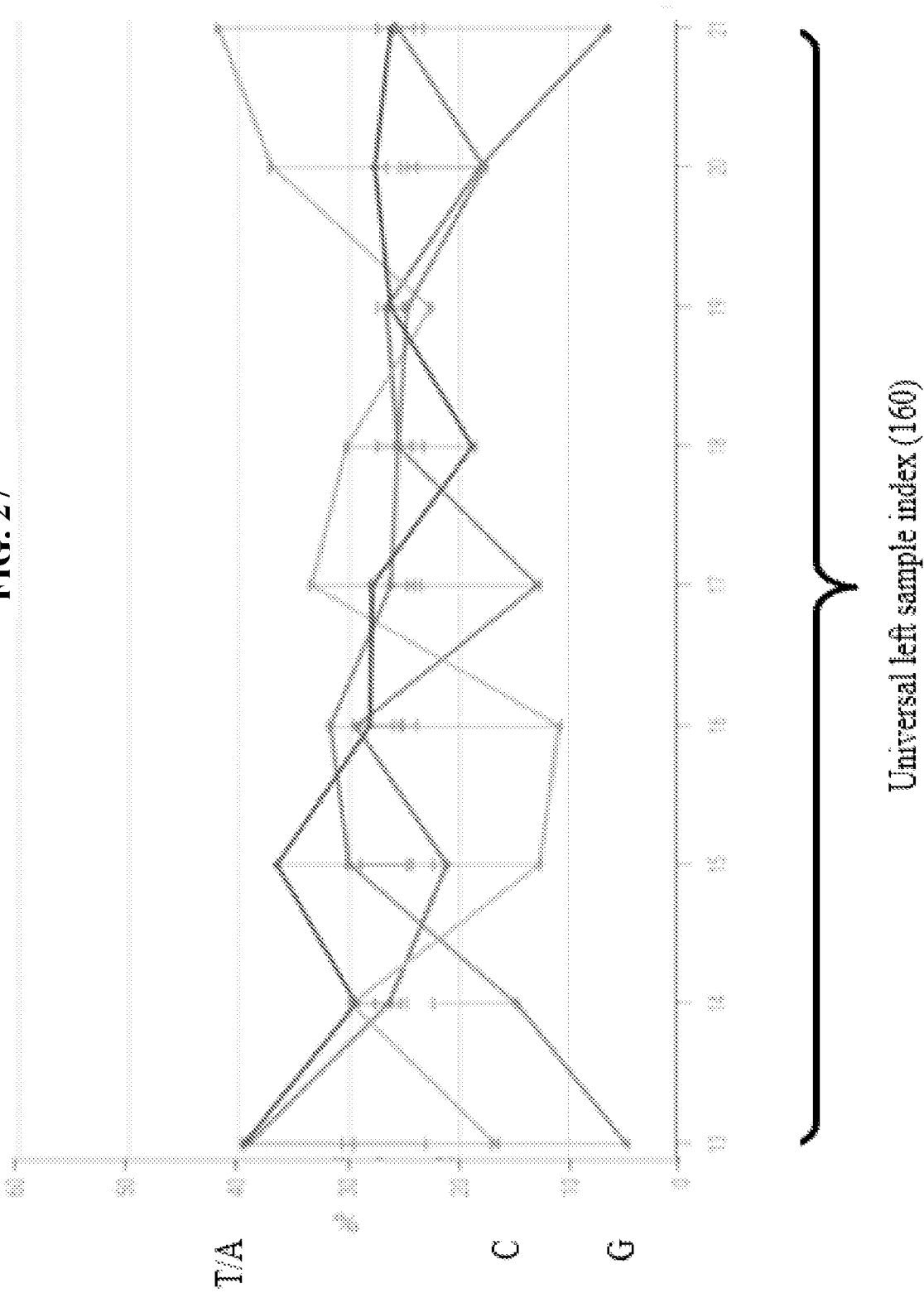


FIG. 27



# INTERNATIONAL SEARCH REPORT

International application No  
**PCT/US2023/063734**

**A. CLASSIFICATION OF SUBJECT MATTER**  
**INV. C12Q1/6855**  
**ADD.**

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
**C12Q**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**EPO-Internal, WPI Data**

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                               | Relevant to claim No.        |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|
| <b>A</b>  | <b>WO 2014/196863 A1 (KEYGENE NV [NL])</b><br><b>11 December 2014 (2014-12-11)</b><br><b>paragraphs [0069], [0101]; claims 1, 49;</b><br><b>figure 5</b><br>-----                                | <b>1-14,</b><br><b>55-59</b> |
| <b>A</b>  | <b>WO 2012/003374 A2 (UNIV LELAND STANFORD</b><br><b>JUNIOR [US]; MYLLYKANGAS SAMUEL [FI] ET</b><br><b>AL.) 5 January 2012 (2012-01-05)</b><br><b>page 2, last paragraph; figure 15</b><br>----- | <b>1-14,</b><br><b>55-59</b> |
| <b>A</b>  | <b>WO 2019/068797 A1 (HOFFMANN LA ROCHE [CH];</b><br><b>ROCHE DIAGNOSTICS GMBH [DE] ET AL.)</b><br><b>11 April 2019 (2019-04-11)</b><br><b>claim 1; figure 4</b><br>-----                        | <b>1-14,</b><br><b>55-59</b> |

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

☒ See patent family annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

**8 July 2023**

Date of mailing of the international search report

**17/07/2023**

Name and mailing address of the ISA/  
European Patent Office, P.B. 5818 Patentlaan 2  
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Fax: (+31-70) 340-3016

Authorized officer

**Aslund, Fredrik**

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/063734

### Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
  - a. ☒ forming part of the international application as filed.
  - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).  
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2023/063734

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|---------------------|----------------------------|---------------------|
| WO 2014196863 A1                          | 11-12-2014          | CA 2913236 A1              | 11-12-2014          |
|                                           |                     | EP 3004381 A1              | 13-04-2016          |
|                                           |                     | JP 2016521557 A            | 25-07-2016          |
|                                           |                     | US 2015284789 A1           | 08-10-2015          |
|                                           |                     | US 2016083788 A1           | 24-03-2016          |
|                                           |                     | WO 2014196863 A1           | 11-12-2014          |
| -----                                     |                     |                            |                     |
| WO 2012003374 A2                          | 05-01-2012          | US 2012003657 A1           | 05-01-2012          |
|                                           |                     | WO 2012003374 A2           | 05-01-2012          |
| -----                                     |                     |                            |                     |
| WO 2019068797 A1                          | 11-04-2019          | CN 111164219 A             | 15-05-2020          |
|                                           |                     | EP 3692166 A1              | 12-08-2020          |
|                                           |                     | JP 7083393 B2              | 10-06-2022          |
|                                           |                     | JP 2020537500 A            | 24-12-2020          |
|                                           |                     | US 2020208208 A1           | 02-07-2020          |
|                                           |                     | WO 2019068797 A1           | 11-04-2019          |
| -----                                     |                     |                            |                     |