What is claimed is:

1. A method for ligation-coupled polymerase chain reaction (PCR), comprising:

(i) providing a partially double-stranded DNA substrate comprising a first strand and a second strand, the partially double-stranded DNA substrate comprising a first 3' overhang, a double-stranded portion, and a second 3' overhang,

the first strand comprising, in a 5' to 3' direction: a first 5' end, a first portion, and a second portion,

the second strand comprising, in a 5' to 3' direction: a second 5' end, a third portion, and a fourth portion of the partially double-stranded DNA substrate,

wherein the first portion of the first strand and the third portion of the second strand are complementary and form the double-stranded portion,

wherein the second portion of the first strand forms the first 3' overhang,

wherein the fourth portion of the second strand forms the second 3' overhang,

wherein the second portion of the first strand and the fourth portion of the second strand each comprise a first common nucleotide sequence positioned at a 5' end of the first 3' overhang and the second 3' overhang, respectively, and

wherein the second portion of the first strand and the fourth portion of the second strand each comprise a second common nucleotide sequence positioned 3' to the first common nucleotide sequence;

(ii) adding a plurality of first indexing primers, a plurality of second indexing primers, a ligase, a DNA polymerase and deoxynucleotide triphosphates (dNTPs) to the partially double-stranded DNA substrate to yield a first reaction mixture, wherein each of the plurality of first indexing primers comprise a first 3' terminal portion complementary to the first common nucleotide sequence, and wherein each of the plurality of second indexing primers comprise a second 3' terminal portion complementary to the first common nucleotide sequence and a first 5' portion positioned 5' to the second 3' terminal portion and complementary to the second common nucleotide sequence;

(iii) incubating the first reaction mixture under a first set of conditions comprising a ligation temperature for a ligation duration, wherein the first set of conditions is sufficient:

a) for the first 3' terminal portion to anneal to the first common nucleotide sequence, and

b) for the ligase to ligate one of the plurality of first indexing primers to the first 5’ end of the first strand and one of the plurality of first indexing primers to the second 5’ end of the second strand to yield a second reaction mixture comprising:

a third strand comprising, in a 5’ to 3’ direction, one of the plurality of first indexing primers, the first portion, and the second portion, and

a fourth strand comprising, in a 5’ to 3’ direction, one of the plurality of first indexing primers, the third portion, and the fourth portion;

(iv) incubating the second reaction mixture under a second set of conditions comprising a first denaturation temperature for a first denaturation duration, a first annealing temperature for a first annealing duration, and a first extension temperature for a first extension duration, wherein the second set of conditions is sufficient:

a) to inactivate the ligase, denature double-stranded DNA, and, optionally, to activate the DNA polymerase,

b) for the second 3’ terminal portion and the first 5’ portion of one of the plurality of second indexing primers to anneal to at least the first common nucleotide sequence and the second common nucleotide sequence of the second portion of the third strand and for the second 3’ terminal portion and the first 5’ portion of one of the plurality of second indexing primers to anneal to at least the first common nucleotide sequence and the second common nucleotide sequence of the fourth portion of the fourth strand, and

c) for the DNA polymerase to extend the one of the plurality of second indexing primers annealed to the first common nucleotide sequence and second common nucleotide sequence of the second portion of the third strand and for the DNA polymerase to extend the one of the plurality of second indexing primers annealed to the first common nucleotide sequence and second common nucleotide sequence of the fourth portion of the fourth strand, to yield a third reaction mixture comprising the third strand, the fourth strand, a fifth strand, and a sixth strand, the fifth strand comprising, in a 5’ to 3’ direction, one of the plurality of second indexing primers, the third portion, and a reverse complement of one of the plurality of first indexing primers, the sixth strand comprising, in a 5’ to 3’ direction, one of the plurality of second indexing primers, the first portion, and the reverse complement of one of the plurality of first indexing primers;

(v) incubating the third reaction mixture under a third set of conditions comprising a second denaturation temperature for a second denaturation duration, a second annealing temperature for a second annealing duration, and a second extension temperature for a second extension duration, wherein the third set of conditions is sufficient

a) to denature double-stranded DNA,

b) for one of the plurality of first indexing primers to anneal to the reverse complement of one of the plurality of first indexing primers of the fifth strand and for one of the plurality of first indexing primers to anneal to the reverse complement of one of the plurality of first indexing primers of the sixth strand, and

c) for the DNA polymerase to extend the one of the plurality of first indexing primers annealed to the reverse complement of one of the plurality of first indexing primers of the fifth strand and the one of the plurality of first indexing primers annealed to the reverse complement of the one of the plurality of first indexing primers of the sixth strand, to yield a fourth reaction mixture comprising the fifth strand, the sixth strand, a seventh strand, and an eighth strand, the seventh strand comprising, in a 5' to 3' direction, one of the plurality of first indexing primers, the first portion, and a reverse complement of one of the plurality of second indexing primers, the eighth strand comprising, in a 5' to 3' direction, one of the plurality of first indexing primers, the third portion, and the reverse complement of one of the plurality of second indexing primers, wherein the seventh strand is complementary to the fifth strand, and wherein the eighth strand is complementary to the sixth strand; and

(vi) incubating the fourth reaction mixture under a fourth set of conditions comprising a third denaturation temperature for a third denaturation duration, a third annealing temperature for a third annealing duration, and a third extension temperature for a third extension duration, wherein the fourth set of conditions is sufficient for at least a portion of the plurality of first indexing primers and at least a portion of the plurality of second indexing primers to amplify the fifth strand and seventh strand and the sixth strand and eighth strand.

2. The method of claim 1, wherein steps (i) through (vi) are performed in a single closed tube.

3. The method of claim 1, wherein the partially double-stranded DNA substrate has a length of about 24 bases to about 6000 bases, wherein the first portion and the third portion of the first strand and the second strand, respectively, each have a length of about 20 bases to about 6000 bases, wherein the second portion of the first strand and the fourth portion of the second strand

each have a length of about 4 bases to about 100 bases, wherein the first common nucleotide sequence has a length of about 1 base to about 50 bases, wherein the second common nucleotide sequence has a length from about 5 bases to about 30 bases, and wherein each of the plurality of first indexing primers has a length from about 20 bases to about 100 bases, and wherein each of the plurality of second indexing primers has a length from about 20 bases to about 100 bases.

~~4. The method of claim 1, wherein the first portion and the third portion of the first strand and the second strand, respectively, each have a length of about 20 bases to about 6000 bases.~~

~~5. The method of claim 1, wherein the second portion of the first strand and the fourth portion of the second strand each have a length of about 4 bases to about 100 bases.~~

~~6. The method of claim 1, wherein the first common nucleotide sequence has a length of about 1 base to about 50 bases.~~

~~7. The method of claim 1, wherein the first common nucleotide sequence has a length of 13 bases.~~

~~8. The method of claim 1, wherein the first common nucleotide sequence comprises the sequence of SEQ ID NO: 127 (5'-AGATCGGAAGAGC-3').~~

~~9. The method of claim 8, wherein the first 3' terminal portion and the second 3' terminal portion each comprise the sequence of SEQ ID NO: 126 (5'-GCTCTTCCGATCT-3').~~

~~10. The method of claim 1, wherein each of the plurality of first indexing primers has a length from about 20 bases to about 100 bases, and wherein each of the plurality of second indexing primers has a length from about 20 bases to about 100 bases.~~

~~11.~~4. The method of claim 1, wherein the first common nucleotide sequence and the first 3' terminal portion have a melting temperature (T_m) greater than the ligation temperature, wherein the ligation temperature is less than a melting temperature (T_m) of the partially double-stranded DNA substrate, wherein a melting temperature (T_m) of the third strand and fourth strand is less than the first denaturing temperature, and wherein the first common nucleotide sequence,~~and wherein the first common nucleotide sequence and~~ the first 3' terminal portion, and the second 3' terminal portion have a T_m less than the first annealing temperature.

~~12. The method of claim 11, wherein the T_m of the first common nucleotide sequence and the first 3' terminal portion is at least 1 °C higher that the ligation temperature, and wherein the T_m of the first common nucleotide sequence and the first 3' terminal portion is at least 1 °C lower than the first annealing temperature.~~

~~13. The method of claim 1, wherein the first common nucleotide sequence and the first 3' terminal portion have a melting temperature (T_m) lower than the first annealing temperature, wherein the first common nucleotide sequence and the second 3' terminal portion have a T_m lower than the first annealing temperature.~~

~~14. The method of claim 1, wherein the second common nucleotide sequence has a length from about 1 base to about 100 bases.~~

~~15. The method of claim 1, wherein the second common nucleotide sequence has a length from about 1 base to about 20 bases.~~

~~16. The method of claim 1, wherein the second common nucleotide sequence has a length of 20 bases.~~

~~17. The method of claim 1, wherein the ligation temperature is at least 1 °C less than a melting temperature (T_m) of the partially double-stranded DNA substrate.~~

~~18. The method of claim 1, wherein the ligation temperature is less than a melting temperature (T_m) of the partially double-stranded DNA substrate.~~

~~19. The method of claim 1, wherein a melting temperature (T_m) of the third strand and fourth strand is less than the first denaturing temperature.~~

~~20.~~5. The method of claim 1, wherein the ligation temperature is from about 25 °C to about 40 °C.

~~21.~~6. The method of claim 1, wherein the ligation duration is from about 5 minutes to about 60 minutes.

~~22. The method of claim 1, wherein the ligation duration is about 20 minutes.~~

~~23.~~7. The method of claim 1, wherein the ligase is a thermolabile ligase capable of ligation in a low magnesium buffer.

~~24.~~8. The method of claim 1, wherein the ligase is T3 DNA ligase.

~~25.~~9. The method of claim 8~~24~~, wherein the ligase is added at about 30 to about 300 enzyme units per 50 µL of the first reaction mixture.

~~26.~~10. The method of claim 1, wherein the ligase is temperature-sensitive, and wherein the first denaturation temperature for the first denaturation duration in step (iv)(a) is sufficient to inactivate the ligase.

~~27.~~11. The method of claim 1, wherein the DNA polymerase is a thermostable DNA polymerase with 3'-5' exonuclease proofreading activity selected from the group consisting of

Kapa HiFi DNA Polymerase (Roche), NEB Q5 DNA Polymerase (NEB), PrimeStar GXL DNA Polymerase (Takara) and High Fidelity DNA Polymerase (Qiagen).

~~28.~~12. The method of claim 1, wherein the DNA polymerase is not active at the ligation temperature.

~~29.~~13. The method of claim 12~~28~~, wherein the DNA polymerase further comprises a hot start antibody or aptamer, and wherein the hot start antibody or aptamer increases an activation temperature of the DNA polymerase.

~~30.~~14. The method of claim 13~~29~~, wherein the DNA polymerase is selected from the group consisting of Kapa HiFi Hot Start DNA Polymerase (Roche), NEB Q5 Hot Start DNA Polymerase (NEB), PrimeStar GXL Hot Start DNA Polymerase (Takara) and High Fidelity Hot Start DNA Polymerase (Qiagen).

~~31.~~15. The method of claim 1, wherein the DNA polymerase is a hot start polymerase, and wherein the first denaturation temperature for the first denaturation duration in step (iv)(a) is sufficient to activate the hot start polymerase.

~~32.~~16. The method of claim 1, wherein a melting temperature (T_m) of each of the plurality of second indexing primers and the second portion or the fourth portion of the first strand and second strand, respectively, is higher than a T_m of each of the plurality of first indexing primers and the second portion or the fourth portion of the first strand and the second strand.

~~33.~~17. The method of claim 1, wherein the first denaturation temperature, the second denaturation temperature, and the third denaturation temperature are each independently about 95 ^{o}C to about 98 ^{o}C, and wherein the first denaturation duration, the second denaturation duration, and the third denaturation duration are each independently from about 30 seconds to about 2 minutes.

~~34. The method of claim 1, wherein the first denaturation duration, the second denaturation duration, and the third denaturation duration are each independently from about 30 seconds to about 2 minutes.~~

~~35.~~18. The method of claim 1, wherein the first annealing temperature, the second annealing temperature, and the third annealing temperature are each independently about 55 ^{o}C to about 65 ^{o}C, and wherein the first annealing duration, the second annealing duration, and the third annealing duration are each independently about 10 seconds to about 60 seconds.

~~36. The method of claim 1, wherein the first annealing duration, the second annealing duration, and the third annealing duration are each independently about 10 seconds to about 60 seconds.~~

~~37.~~19. The method of claim 1, wherein the first extension temperature, the second extension temperature, and the third extension temperature are each independently about 62 ^{o}C to about 72 ^{o}C, and wherein the first extension duration, the second extension duration, and the third extension duration are each independently about 30 seconds to about 5 minutes.

~~38. The method of claim 1, wherein the first extension duration, the second extension duration, and the third extension duration are each independently about 30 seconds to about 5 minutes.~~

~~39.~~20. The method of claim 1, wherein the plurality of first indexing primers and the plurality of second indexing primers are each independently added to the first reaction mixture at about 100 nM to about 1 µM.

~~40. The method of claim 1, wherein the plurality of first indexing primers and the plurality of second indexing primers are each independently added to the first reaction mixture at about 100 nM to about 200 nM.~~

~~41. The method of claim 1, wherein step (ii) further comprises adding a blocker oligonucleotide to the first reaction mixture, wherein the blocker oligonucleotide comprises a 5' portion that is complementary to at least a portion of the second 3' terminal portion of each of the plurality of second indexing primers, wherein a melting temperature (T_m) of the blocker oligonucleotide and each of the plurality of second indexing primers is greater than the ligation temperature and less than the first annealing temperature and the third annealing temperature.~~

~~42. The method of claim 41, wherein the T_m of the blocker oligonucleotide and each of the plurality of second indexing primers is greater than a T_m of the blocker oligonucleotide and each of the plurality of first indexing primers.~~

~~43. The method of claim 41, wherein the blocker oligonucleotide is added in an amount sufficient to inhibit ligation of each of the plurality of second indexing primers to the first 5' end of the first strand and to the second 5' end of the second strand.~~

~~44. The method of claim 41, wherein the blocker oligonucleotide is added in an amount that is 1X, 1.5X, 2X, 4X, or 6X an amount of the plurality of second indexing primers.~~

~~45. The method of claim 41, wherein the blocker oligonucleotide is pre-annealed to each of the plurality of second indexing primers.~~

46. The method of claim 41, wherein the blocker oligonucleotide comprises a first additional portion positioned 3' to the 5' portion which is complementary to each of the plurality of second indexing primers and not complementary to each of the plurality of first indexing primers.

47. The method of claim 46, wherein a melting temperature (T_m) between the first additional portion and 5' portion and each of the plurality of second indexing primers is about the same or greater than a melting temperature between the 5' portion and each of the plurality of first indexing primers and each of the plurality of second indexing primers.

48. The method of claim 47, wherein the T_m between the first additional portion and the 5' portion and each of the plurality of second indexing primers is at least 1 ^{o}C greater than a melting temperature between the 5' portion and each of the plurality of first indexing primers and each of the plurality of second indexing primers.

49. The method of any of claims 46-48, wherein the blocker oligonucleotide further comprises a second additional portion positioned between the 5' portion and the first additional portion, and wherein the second additional portion is not complementary to each of the plurality of first indexing primers and is not complementary to each of the plurality of second indexing primers.

50. The method of claim 49, wherein the second additional portion has a length from about 1 to about 30 bases.

51. The method of claim 41, wherein the blocker oligonucleotide comprises a sequence that is not complementary to each of the second indexing primers.

52. The method of claim 51, wherein the sequence that is not complementary to each of the plurality of second indexing primers has a length from about 1 to about 30 bases.

53. The method of claim 41, wherein the blocker oligonucleotide further comprises a 3' modification to block polymerase extension.

54. The method of claim 53, wherein the 3' modification to block polymerase extension is selected from the group consisting of a C3 carbon spacer, hexanediol, spacer 9, spacer 18, phosphate, 2',3'-dideoxynucleosides ddA, ddT, ddC and ddG, 3'-deoxynucleosides 3'-A, 3'-T, 3'-C and 3'-G, RNA nucleotides such as rU, 3-O-methyl nucleotides, or a DNA sequence that is not complementary to the adjacent primer sequence such as poly-T, poly-A, poly-C and poly-G and additionally comprises nuclease resistant linkages to prevent proofreading polymerase 3'-5' exonuclease activity from removing the DNA sequence that is not complementary to the adjacent primer sequence.

55. The method of claim 41, wherein a melting temperature (T_m) between the blocker oligonucleotide and each of the plurality of first indexing primers is less than a T_m between the blocker oligonucleotide and each of the plurality of second indexing primers.

56. The method of claim 55, wherein the T_m between the blocker oligonucleotide and each of the plurality of first indexing primers is less than a T_m between the blocker oligonucleotide and each of the plurality of second indexing primers by at least 5 ^{o}C.

57. The method of claim 41, wherein the 5' portion of the blocker oligonucleotide comprises the sequence of SEQ ID NO: 127.

58. The method of any of claims 46-50, wherein the blocker oligonucleotide further comprises a 3' hydroxyl group and a hairpin portion positioned 3' to the first additional portion, wherein the hairpin portion comprises a first hairpin sequence and a second hairpin sequence, wherein the first hairpin sequence is positioned 5' to the second hairpin sequence, wherein the first hairpin sequence and the second hairpin sequence are complementary, and wherein the hairpin portion has a melting temperature greater than the first annealing temperature, the second annealing temperature, and the third annealing temperature.

59. The method of claim 58, wherein the hairpin portion further comprises a third hairpin sequence positioned between the first hairpin sequence and the second hairpin sequence.

60. The method of claim 59, wherein the third hairpin sequence forms a loop sufficient to allow formation of a stable stem-loop structure by the hairpin portion and the first additional portion.

61. The method of claim 60, wherein the third hairpin sequence has a length from about 4 bases to about 20 bases.

62. The method of claim 58, wherein a melting temperature of the hairpin portion is greater than a melting temperature between the 5' portion and each of the plurality of second indexing primers, the additional portion and each of the plurality of second indexing primers or both.

63. The method of any of claims 58-60, wherein the second set of conditions is further sufficient for the DNA polymerase to extend a 3' end of the blocker oligonucleotide to yield an extended hairpin blocker.

64. The method of claim 63, wherein the extended hairpin blocker has a melting temperature (T_m) greater than the second annealing temperature and the third annealing temperature.

65. The method of claim 63, wherein the extended hairpin blocker has a stabilized secondary structure.

66. The method of claim 63, wherein a melting temperature (T_m) of the extended hairpin blocker is higher than a T_m of the 5' portion and the first additional portion to each of the plurality of second indexing primers.

67. The method of claim 1, wherein each of the plurality of first indexing primers further comprises the sequence of SEQ ID NO: 87.

68. The method of claim 1, wherein each of the plurality of second indexing primers further comprises the sequence of SEQ ID NO: 78.

69. The method of any of the preceding claims further comprising sequencing the fifth strand and seventh strand or the sixth strand and the eighth strand.

70. The method of any of the preceding claims, wherein each of the plurality of first indexing primers comprises a first 5' terminal portion and each of the plurality of second indexing primers further comprises a second 5' terminal portion, wherein each of the first 5' terminal portion and the second 5' terminal portion comprise, in a 5' to 3' direction, a first sequence comprising two or more deoxynucleotides and a second sequence comprising three or more ribonucleotides, wherein the DNA polymerase has 3' to 5' exonuclease activity, whereby the fifth strand and sixth strand further comprise the second 5' terminal portion at a 5' end of the fifth strand and sixth strand, and whereby the seventh strand and eighth strand further comprise the first 5' terminal portion at a 5' end of the seventh strand and eighth strand, and whereby the fifth strand and seventh strand can form a first double-stranded product having a first 5' overhang and a second 5' overhang, and whereby the sixth strand and eighth strand can form a second double-stranded product having a third 5' overhang and a fourth 5' overhang, the method further comprising:

adding a probe complementary to each of the first 5' overhang, second 5' overhang, third 5' overhang and fourth 5' overhang in an amount sufficient to yield a target molar quantity of the fifth strand, sixth strand, seventh strand and eighth strand, and a second ligase where the fifth strand, sixth strand, seventh strand and eighth strand are present in an amount greater than the target molar quantity, wherein the probe comprises a modification to provide resistance to digestion by an enzyme with 3' exoenuclease activity;

incubating the fifth strand, sixth strand, seventh strand, eighth strand, second ligase and probe under conditions sufficient for the probe to ligate to the target molar quantity of the fifth

strand, sixth strand, seventh strand and eighth strand to yield a pre-normalization reaction mixture;

adding an enzyme with 3' exonuclease activity to the pre-normalization reaction mixture;

incubating the pre-normalization reaction mixture and enzyme with exonuclease activity under conditions sufficient for the enzyme with 3' exonuclease activity to digest the fifth strand sixth strand, seventh strand, and eighth strand not ligated to the probe, to yield a normalized next generation sequencing (NGS) library.

71. The method of claim 70, further comprising sequencing the normalized NGS library.

72. A method for ligation-coupled PCR, comprising:

(i) providing a partially double-stranded DNA substrate comprising a first strand and a second strand, the partially double-stranded DNA substrate comprising a first 3' overhang, a double-stranded portion, and a second 3' overhang,

the first strand comprising, in a 5' to 3' direction, a first 5' end, a first portion, and a second portion,

the second strand comprising, in a 5' to 3' direction, a second 5' end, a third portion, and a fourth portion,

wherein the first portion of the first strand and the third portion of the second strand are complementary and form the double-stranded portion,

wherein the second portion of the first strand forms the first 3' overhang,

wherein the fourth portion of the second strand forms the second 3' overhang,

wherein the second portion of the first strand and the fourth portion of the second strand each comprise a first common nucleotide sequence positioned at a 5' end of the first 3' overhang and the second 3' overhang, respectively, and

wherein the second portion of the first strand and the fourth portion of the second strand each comprise a second common nucleotide sequence positioned 3' to the first common nucleotide sequence;

(ii) adding a plurality of first indexing primers, a plurality of second indexing primers, a plurality of 5' adapters, a ligase, a DNA polymerase and deoxynucleotide triphosphates (dNTPs) to the partially double-stranded DNA substrate to yield a first reaction mixture, wherein each of the plurality of first indexing primers comprise a first 3' terminal portion that is not complementary to the first common nucleotide sequence, wherein each of the plurality of second indexing primers comprises a second 3' terminal portion that is not complementary to the first common nucleotide sequence, wherein the second 3' terminal sequence is complementary to the second common nucleotide sequence, and wherein each of the plurality of 5' adapters comprises a third 3' terminal sequence complementary to the first common nucleotide sequence, a first 5' portion positioned 5' to the third 3' terminal sequence which comprises at least a portion of the first 3' terminal portion of each of the plurality of first indexing primers, a second 5' portion positioned 5' to the first 5' portion and complementary to the first 5' portion, and a replication blocker capable of blocking the DNA polymerase positioned at a 5' end of the first 5' portion, and wherein the 5' adapter can form a hairpin formed by annealing of the first 5' portion to the second 5' portion;

(iii) incubating the first reaction mixture under a first set of conditions comprising a ligation temperature for a ligation duration, sufficient: 1) for the third 3' terminal portion to anneal to the first common nucleotide sequence, and 2) for the ligase to ligate one of the plurality of 5' adapters to the first 5' end of the first strand and the second 5' end of the second strand, to yield a second reaction mixture comprising:

a third strand comprising, in a 5' to 3' direction, one of the plurality of 5' adapters, the first portion, and the second portion, and

a fourth strand comprising, in a 5' to 3' direction, one of the plurality of 5' adapters, the third portion, and the fourth portion;

(iv) incubating the second reaction mixture under a second set of conditions comprising a first denaturation temperature for a first denaturation duration, a first annealing temperature for a first annealing duration, and a first extension temperature for a first extension duration, sufficient:

a) to inactivate the ligase, denature double-stranded DNA and, optionally, to activate the DNA polymerase,

b) for the second 3' terminal portion of one of the plurality of second indexing primers to anneal to the second common nucleotide sequence of the second portion of the third strand and for one of the plurality of second indexing primers to anneal to the second common nucleotide sequence of the fourth portion of the fourth strand,

c) for the DNA polymerase to extend the one of the plurality of second indexing primers annealed to the second common nucleotide sequence of the second portion of the third strand and to extend the one of the plurality of second indexing primers annealed to the second common nucleotide sequence of the fourth portion of the fourth strand, to yield a third reaction mixture comprising the third strand, the fourth strand, a fifth strand, and a sixth strand, the fifth strand comprising, in a 5' to 3' direction, one of the plurality of second indexing primers, the third portion, a reverse complement of the first common nucleotide sequence, and a reverse complement of the first 5' portion of one of the plurality of 5' adapters, and the sixth strand comprising, in a 5' to 3' direction, one of the plurality of second indexing primers, the first portion, the reverse complement of the first common nucleotide sequence, and the reverse complement of the first 5' portion of one of the plurality of 5' adapters,

(v) incubating the third reaction mixture under a third set of conditions comprising a second denaturation temperature for a second denaturation duration, a second annealing temperature for a second annealing duration, a second extension temperature for a second extension duration, sufficient:

a) to denature any double-stranded DNA,

b) for the first 3' terminal portion of one of the plurality of first indexing primers to anneal to the reverse complement of the first 5' portion of one of the plurality of 5' adapters of the fifth strand and one of the plurality of first indexing primers to anneal to the reverse complement of the first 5' portion of one of the plurality of 5' adapters of the sixth strand,

c) for the DNA polymerase to extend the one of the plurality of first indexing primers annealed to the reverse complement of the first 5' portion of one of the plurality of 5'

adapters of the fifth strand and to extend the one of the plurality of first indexing primers annealed to the reverse complement of the first 5' portion of one of the plurality of 5' adapters of the sixth strand, to yield a fourth reaction mixture comprising the fifth strand, the sixth strand, a seventh strand and an eighth strand, the seventh strand comprising, in a 5' to 3' direction, one of the plurality of first indexing primers, the first common nucleotide sequence, the first portion, and and a reverse complement of one of the plurality of second indexing primers, and the eighth strand comprising, in a 5' to 3' direction, one of the plurality of first indexing primers, the first common nucleotide sequence, the third portion, the reverse complement of one of the plurality of second indexing primers;

(vi) incubating the fourth reaction mixture under a fourth set of conditions comprising a third denaturation temperature for a third denaturation duration, a third annealing temperature for a third annealing duration, and a third extension temperature for a third extension duration, sufficient:

a) to denature any double-stranded DNA,

b) for one of the plurality of second indexing primers to anneal to the reverse complement of one of the plurality of second indexing primers of the seventh strand and for one of the plurality of second indexing primers to anneal to the reverse complement of one of the plurality of second indexing primers of the eighth strand, and

c) for the DNA polymerase to extend the one of the plurality of second indexing primers annealed to each of the seventh strand and eighth strand to yield a fifth reaction mixture comprising the seventh strand, the eighth strand, a ninth strand and a tenth strand, the ninth strand comprising, in a 5' to 3' direction, one of the plurality of second indexing primers, the third portion, the reverse complement of the first common nucleotide sequence, and a reverse complement of one of the plurality of first indexing primers, and the tenth strand comprising, in a 5' to 3' direction, one of the plurality of second indexing primers, the first portion, the reverse complement of the first common nucleotide sequence, and the reverse complement of one of the plurality of first indexing primers, wherein the seventh strand and ninth strand are complementary and wherein the eighth strand and tenth strand are complementary; and

(vii) incubating the fifth reaction mixture under a fifth set of conditions comprising a fourth denaturation temperature for a fourth denaturation duration, a fourth annealing temperature

for a fourth annealing duration, and a fourth extension temperature for a fourth extension duration, sufficient for at least a portion of the plurality of first indexing primers, at least a portion of the plurality of second indexing primers and the DNA polymerase to amplify the seventh strand and ninth strand and eighth strand and tenth strand.

73. The method of claim 72, wherein steps (i)-(vii) are performed in a single closed tube.

74. The method of claim 72, wherein the first 5' portion and the second 5' portion each have a length of about 12 bases to about 20 bases.

75. The method of claim 72, wherein each of the plurality of 5' adapters further comprises an intervening sequence between the first 5' portion and the second 5' portion.

76. The method of claim 75, wherein the intervening sequence has a length of about 4 bases to about 20 bases.

77. The method of claim 72, wherein the replication block is selected from the group consisting of a stable abasic site, a C3 spacer, hexandiol, Spacer 9, Spacer 18, 3 or more rU bases, and 2'-O-methyl RNA bases.

78. The method of claim 72, wherein each of the plurality of 5' adapters has a length of about 25 bases to about 100 bases.

79. The method of claim 72, wherein the partially-double stranded DNA substrate has a length of about 24 bases to about 6000 bases.

80. The method of claim 72, wherein the first portion and the third portion of the first strand and the second strand, respectively, each have a length of about 20 bases to about 6000 bases.

81. The method of claim 72, wherein the second portion of the first strand and the fourth portion of the second strand each have a length of about 4 bases to about 100 bases.

82. The method of claim 72, wherein the first common nucleotide sequence has a length of about 1 base to about 50 bases.

83. The method of claim 72, wherein the first common nucleotide sequence has a length of 13 bases.

84. The method of claim 72, wherein the first common nucleotide sequence comprises the sequence of SEQ ID NO: 127.

85. The method of claim 84, wherein the third 3' terminal portion of each of the plurality of 5' adapters comprises the sequence of SEQ ID NO: 126.

86. The method of claim 72, wherein each of the plurality of first indexing primers has a length from about 20 bases to about 100 bases, and wherein each of the plurality of second indexing primers has a length from about 20 bases to about 100 bases.

87. The method of claim 72, wherein the first common nucleotide sequence and the third 3' terminal portion have a melting temperature greater than the ligation temperature.

88. The method of claim 72, wherein the second 3' terminal portion of each of the plurality of second indexing primers and the second common nucleotide sequence has a melting temperature greater than the first annealing temperature.

89. The method of claim 72, wherein the melting temperature of the first common nucleotide sequence and the third 3' terminal portion is at least 1 °C higher than the ligation temperature, and wherein the melting temperature of the first common nucleotide sequence and the third 3' terminal portion is at least 1 °C lower than the first annealing temperature.

90. The method of claim 72, wherein the second common nucleotide sequence has a length from about 1 base to about 100 bases.

91. The method of claim 72, wherein the second common nucleotide sequence a has length from about 1 base to about 20 bases.

92. The method of claim 72, wherein the second common nucleotide sequence comprises the sequence of SEQ ID NO: 95.

93. The method of claim 72, wherein the ligation temperature is less than a melting temperature of the partially double-stranded DNA substrate.

94. The method of claim 72, wherein a melting temperature of the third strand and fourth strand is less than the first denaturing temperature.

95. The method of claim 72, wherein the ligation temperature is from about 25 °C to about 40 °C.

96. The method of claim 72, wherein the ligation duration is from about 5 minutes to about 60 minutes.

97. The method of claim 72, wherein the ligation duration is about 20 minutes.

98. The method of claim 72, wherein the ligase is a thermolabile ligase capable of ligation in a low magnesium buffer.

99. The method of claim 72, wherein the ligase is T3 DNA ligase.

~~100. The method of claim 99, wherein the ligase is added at about 30 to about 300 enzyme units per 50 µL of the first reaction mixture.~~

~~101. The method of claim 72, wherein the ligase is temperature-sensitive, and wherein the first denaturation temperature for the first denaturation duration is sufficient to inactivate the ligase.~~

~~102. The method of claim 72, wherein the DNA polymerase is selected from the group consisting of Kapa HiFi DNA Polymerase (Roche), NEB Q5 DNA Polymerase (NEB), PrimeStar GXL DNA Polymerase (Takara) and High Fidelity DNA Polymerase (Qiagen).~~

~~103. The method of claim 72, wherein the DNA polymerase is not active at the ligation temperature.~~

~~104. The method of claim 103, wherein the DNA polymerase further comprises a hot start antibody or aptamer, and wherein the hot start antibody or aptamer increases an activation temperature of the DNA polymerase.~~

~~105. The method of claim 104, wherein the DNA polymerase is selected from the group consisting of Kapa HiFi Hot Start DNA Polymerase (Roche), NEB Q5 D Hot Start NA Polymerase (NEB), PrimeStar GXL Hot Start DNA Polymerase (Takara) and High Fidelity Hot Start DNA Polymerase (Qiagen).~~

~~106. The method of claim 72, wherein the DNA polymerase is a hot start polymerase, and wherein the first denaturation temperature for the first denaturation duration is sufficient to activate the hot start polymerase.~~

~~107. The method of claim 72, wherein the first denaturation temperature, the second denaturation temperature, the third denaturation temperature, and the fourth denaturation temperature are each independently about 95 °C to about 98 °C.~~

~~108. The method of claim 72, wherein the first denaturation duration, the second denaturation duration, the third denaturation duration, and the fourth denaturation duration are each independently from about 30 seconds to about 2 minutes.~~

~~109. The method of claim 72, wherein the plurality of first indexing primers and the plurality of second indexing primers are each independently added to the first reaction mixture at about 100 nM to about 1 µM.~~

110. The method of claim 72, wherein the plurality of first indexing primers and the plurality of second indexing primers are each independently added to the first reaction mixture at about 100 nM to about 200 nM.

111. The method of claim 72, wherein the first annealing temperature, the second annealing temperature, the third annealing temperature, and the fourth annealing temperature are each independently about 55 ºC to about 65 ºC.

112. The method of claim 72, wherein the first annealing duration, the second annealing duration, the third annealing duration, and the fourth annealing duration are each independently about 10 seconds to about 60 seconds.

113. The method of claim 72, wherein the first extension temperature, the second extension temperature, the third extension temperature, and the fourth extension temperature are each independently about 60 ºC to about 72 ºC.

114. The method of claim 72, wherein the first extension duration, the second extension duration, the third extension duration, and the fourth extension duration are each independently about 30 seconds to about 5 minutes.

115. The method of claim 72, wherein each of the plurality of first indexing primers further comprises the sequence of SEQ ID NO: 87.

116. The method of claim 72, wherein each of the plurality of second indexing primers further comprises the sequence of SEQ ID NO: 78.

117. The method of any of claims 72-116, further comprising sequencing the seventh strand and the ninth strand or the eighth strand and the tenth strand.

118. The method of any of claims 72-117, wherein each of the plurality of first indexing primers comprises a first 5' terminal portion and each of the plurality of second indexing primers further comprises a second 5' terminal portion, wherein each of the first 5' terminal portion and the second 5' terminal portion comprise, in a 5' to 3' direction, a first sequence comprising two or more deoxynucleotides and a second sequence comprising three or more ribonucleotides, wherein the DNA polymerase has 3' to 5' exonuclease activity, whereby the seventh strand and eighth strand further comprise the first 5' terminal portion at a 5' end of the seventh strand and eighth strand, and whereby the ninth strand and tenth strand further comprise the second 5' terminal portion at a 5' end of the ninth strand and tenth strand, and whereby the seventh strand and ninth strand can form a first double-stranded product having a first 5' overhang and a second 5'

overhang, and whereby the eighth strand and tenth strand can form a second double-stranded product having a third 5’ overhang and a fourth 5’ overhang, the method further comprising:

adding a probe complementary to each of the first 5’ overhang, second 5’ overhang, third 5’ overhang and fourth 5’ overhang in an amount sufficient to yield a target molar quantity of the seventh strand, eighth strand, ninth strand, and tenth strand, and a second ligase where the seventh strand, eighth strand, ninth strand, and tenth strand are present in an amount greater than the target molar quantity, wherein the probe comprises a modification to provide resistance to digestion by an enzyme with 3’ exonuclease activity;

incubating the seventh strand, eighth strand, ninth strand, and tenth strand, ligase and probe under conditions sufficient for the probe to ligate to the target molar quantity of the seventh strand, eighth strand, ninth strand, and tenth strand to yield a pre-normalization reaction mixture;

adding an enzyme with 3’ exonuclease activity to the pre-normalization reaction mixture;

incubating the pre-normalization reaction mixture and enzyme with exonuclease activity under conditions sufficient for the enzyme with 3’ exonuclease activity to digest the seventh strand, eighth strand, ninth strand, and tenth strand not ligated to the probe, to yield a normalized next generation sequencing (NGS) library.

119. The method of any of the preceding claims, further comprising prior to step (i):

providing a starting double-stranded DNA substrate molecule comprising a first starting strand and a second starting strand, wherein the first starting strand of the starting double-stranded DNA substrate molecule includes a universal primer sequence at a 5’ end of the first starting strand, wherein the universal primer sequence comprises dU bases internal to or at a 3’ end of the universal primer sequence, wherein the second starting strand of the starting double-stranded DNA substrate molecule includes the universal primer sequence at a 5’ end of the second starting strand;

adding an enzyme capable of cleaving the dU bases of the starting double-stranded DNA substrate molecule; and

incubating the enzyme capable of cleaving the dU bases and starting double-stranded DNA substrate molecule under conditions sufficient to yield the partially double-stranded DNA substrate of step (i).

120. The method of claim 119, wherein the enzyme capable cleaving the dU bases is a combination of uracil DNA glycosylase and Endonuclease VIII.

121. The method of any of claims 1-118, further comprising prior to step (i):

providing a starting double-stranded DNA substrate molecule comprising a first starting strand and a second starting strand, wherein the first starting strand of the starting double-stranded DNA substrate molecule comprises a first 5' cleavable sequence comprising ribonucleotides internal to or at a 3' end of the first 5' cleavable sequence, wherein the second starting strand of the double-stranded DNA substrate molecule comprises a second 5' cleavable sequence comprising ribonucleotides internal to or at a 3' end of the second 5' cleavable sequence;

adding an enzyme capable of cleaving the ribonucleotides of the first 5' cleavable sequence and the second 5' cleavable sequence;

incubating the enzyme capable of cleaving the ribonucleotides of the first 5' cleavable sequence and the second 5' cleavable sequence and the and starting double-stranded DNA substrate molecule under conditions sufficient to yield the partially double-stranded DNA substrate of step (i).

122. The method of claim 121, wherein the enzyme capable of cleaving the ribonucleotides of the first 5' cleavable sequence and the second 5' cleavable sequence is RNase H.

123. The method of any of claims 1-118, further comprising prior to step (i):

providing a starting double-stranded DNA substrate molecule comprising a first starting strand and a second starting strand, wherein the first starting strand of the starting double-stranded DNA substrate molecule comprises a first 5' cleavable sequence comprising inosine bases internal to or at a 3' end of the first 5' cleavable sequence, wherein the second starting strand of the double-stranded DNA substrate molecule comprises a second 5' cleavable sequence comprising inosine bases internal to or at a 3' end of the second 5' cleavable sequence;

adding an enzyme capable of cleaving the inosine bases of the first 5' cleavable sequence and the second 5' cleavable sequence;

~~incubating the enzyme capable of cleaving the inosine bases of the first 5' cleavable sequence and the second 5' cleavable sequence and the starting double-stranded DNA substrate molecule under conditions sufficient to yield the partially double-stranded DNA substrate of step (i)~~

~~124. The method of claim 123, wherein the enzyme capable of cleaving the inosine bases is Endonuclease V.~~

~~125. The method of any of claims 1-118, further comprising:~~

~~providing a fragmented DNA substrate molecule;~~

~~performing end repair to yield a blunt-ended, double-stranded starting DNA substrate molecule comprising a first starting strand and a second starting strand, each of the first starting strand and the second starting strand comprising a 5' end and a 3' end;~~

~~adding a plurality of 3' adapter strands comprising a 5' phosphate, each 3' adapter strand annealed to a complementary strand comprising dU bases and a 3' blocking group, and a ligase to the blunt-ended, double-stranded starting DNA substrate molecule;~~

~~incubating the plurality of 3' adapter strands annealed to complementary strands comprising dU bases, the ligase, and the blunt-ended, double-stranded starting DNA substrate molecule under conditions sufficient to ligate one of the plurality of 3' adapter strands to each 3' end of first strand and second strand of the blunt-ended starting DNA substrate molecule and leave a nick between each complementary strand and each 5' end of the first starting strand and second starting strand, to yield a ligated, double-stranded substrate molecule;~~

~~optionally, purifying the ligated, double-stranded substrate molecule;~~

~~adding to the ligated, double-stranded substrate molecule an enzyme capable of cleaving the dU bases of the ligated, double-stranded substrate molecule; and~~

~~incubating the enzyme capable of cleaving the dU bases and the ligated, double-stranded substrate molecule under conditions sufficient to cleave the complementary strands to yield the partially double-stranded DNA substrate of step (i).~~

~~126. The method of claim 125, wherein the enzyme capable of cleaving the dU bases is a combination of uracil DNA glycosylate and Endonuclease VIII.~~

127. The method of any of claims 1-118, further comprising:

providing a fragmented DNA substrate molecule;

performing end repair to yield a blunt-ended, double-stranded starting DNA substrate molecule comprising a first starting strand and a second starting strand, each of the first starting strand and the second starting strand comprising a 5' end and a 3' end;

adding a plurality of 3' adapter strands comprising a 5' phosphate, each 3' adapter strand annealed to a complementary strand comprising ribonucleotides and a 3' blocking group, and a ligase to the blunt-ended, double-stranded starting DNA substrate molecule;

incubating the plurality of 3' adapter strands annealed to complementary strands comprising ribonucleotides, the ligase, and the blunt-ended, double-stranded starting DNA substrate molecule under conditions sufficient to ligate one of the plurality of 3' adapter strands to each 3' end of first starting strand and second starting strand of the blunt-ended starting DNA substrate molecule and leave a nick between each complementary strand and each 5' end of the first starting strand and second starting strand, to yield a ligated, double-stranded substrate molecule ;

optionally purifying the ligated, double-stranded substrate molecule;

adding to the ligated, double-stranded substrate molecule an enzyme capable of cleaving the ribonucleotides of the ligated, double-stranded substrate molecule; and

incubating the enzyme capable of cleaving the ribonucleotides and the ligated, double-stranded substrate molecule under conditions sufficient to cleave the complementary strands to yield the partially double-stranded DNA substrate of step (i).

128. The method of claim 127, wherein the enzyme capable of cleaving the ribonucleotides is RNase H.

129. The method of any of claims 1-118, further comprising:

providing a fragmented DNA substrate molecule;

performing end repair to yield a blunt-ended, double-stranded starting DNA substrate molecule comprising a first strand and a second strand, each of the first strand and the second strand comprising a 5’ end and a 3’ end;

adding a plurality of 3’ adapter strands comprising a 5’ phosphate, each 3’ adapter strand annealed to a complementary strand comprising inosine bases and a 3’ blocking group, and a ligase to the blunt-ended, double-stranded starting DNA substrate molecule;

incubating the plurality of 3’ adapter strands annealed to complementary strands comprising inosine bases, the ligase, and the blunt-ended, double-stranded starting DNA substrate molecule under conditions sufficient to ligate one of the plurality of 3’ adapter strands to each 3’ end of first strand and second strand of the blunt-ended starting DNA substrate molecule and leave a nick between each complementary strand and each 5’ end of the first and second strand, to yield a ligated, double-stranded substrate molecule ;

optionally purifying the ligated, double-stranded substrate molecule;

adding to the ligation-PCR reaction mixture an enzyme capable of cleaving the inosine bases of the ligated, double-stranded substrate molecule; and

incubating the enzyme capable of cleaving the inosine bases and the ligated, double-stranded substrate molecule under conditions sufficient to cleave the complementary strands to yield the partially double-stranded DNA substrate of step (i).

130. The method of claim 129, wherein the enzyme capable of cleaving the inosine bases is Endonuclease V.

131. The method of any of claims 1-118, further comprising:

providing a fragmented DNA substrate molecule;

performing end repair and A-tailing of the fragmented DNA substrate molecule to yield a single-A-tailed double-stranded starting DNA substrate molecule comprising a first starting strand and a second starting strand, each of the first starting strand and the second starting strand comprising a 5’ end and a 3’ end, the single-A-tailed double-stranded starting DNA substrate molecule comprising a single-A base overhang at each 3’ end;

adding a plurality of 3’ adapter strands comprising a 5’ phosphate, each 3’ adapter strand annealed to a complementary strand comprising a 3’ terminal dU base and a 3’ hydroxyl group, and a ligase to the single-A-tailed double-stranded starting DNA substrate molecule;

incubating the plurality of 3’ adapter strands annealed to complementary strands comprising a 3’ terminal dU base, the ligase, and the single-A-tailed double-stranded starting DNA substrate molecule under conditions sufficient to ligate one of the plurality of 3’ adapter strands to each single A base overhang and to ligate one of the complementary strands to each 5’ end of the single-A-tailed double-stranded starting DNA substrate molecule, to yield a ligated, double-stranded substrate molecule;

optionally purifying the ligated, double-stranded substrate molecule;

adding an enzyme capable of cleaving dU bases to the ligated, double-stranded substrate molecule; and

incubating the enzyme capable of cleaving dU bases and the ligated, double-stranded substrate molecule under conditions sufficient to cleave the complementary strands to yield the partially double-stranded DNA substrate of step (i).

132. The method of claim 131, wherein the enzyme capable of cleaving the dU bases is a combination of uracil DNA glycosylate and Endonuclease VIII.

133. The method of claim 131, wherein the complementary strand comprises additional dU bases internal to the complementary strand.

134. The method of any of claims 1-118, further comprising:

providing a fragmented DNA substrate molecule;

performing end repair and A-tailing of the fragmented DNA substrate molecule to yield a single-A-tailed double-stranded starting DNA substrate molecule comprising a first starting strand and a second starting strand, each of the first starting strand and the second starting strand comprising a 5’ end and a 3’ end, the single-A-tailed double-stranded starting DNA substrate molecule comprising a single-A base overhang at each 3’ end;

adding a plurality of 3’ adapter strands comprising a 5’ phosphate, each 3’ adapter strand annealed to a complementary strand comprising a 3’ terminal T base, 3’ hydroxyl group, and dU bases, and a ligase to the single-A-tailed double-stranded starting DNA substrate molecule;

incubating the plurality of 3’ adapter strands annealed to complementary strands comprising a 3’ terminal T base, the ligase, and the single-A-tailed double-stranded starting DNA substrate molecule under conditions sufficient to ligate one of the plurality of 3’ adapter strands to each single A base overhang and to ligate one of the complementary strands to each 5’ end of the single-A-tailed double-stranded starting DNA substrate molecule, to yield a ligated, double-stranded substrate molecule;

optionally purifying the ligated, double-stranded substrate molecule;

adding an enzyme capable of cleaving the dU bases to the ligated, double-stranded substrate molecule; and

incubating the enzyme capable of cleaving the dU bases and the ligated, double-stranded substrate molecule under conditions sufficient to cleave the complementary strands to yield the partially double-stranded DNA substrate of step (i).

135. The method of claim 134, wherein the enzyme capable of cleaving the dU bases is a combination of uracil DNA glycosylate and Endonuclease VIII.

136. The method of any of claims 125-135, further comprising fragmenting target DNA to yield the fragmented DNA substrate molecule.

137. The method of any of the preceding claims, where no purification is performed between steps (i)-(vi) or (i)-(vii).

138. A method for ligation-coupled polymerase chain reaction (PCR), comprising:

(i) providing a double-stranded DNA substrate comprising a first strand and a second strand, the first strand comprising a first 3’ terminal portion and a first 5’ terminal portion and the second strand comprising a second 3’ terminal portion and a second 5’ terminal portion;

(ii) adding a ligase, a first oligonucleotide, a second oligonucleotide, a third oligonucleotide, a first primer, a DNA polymerase and deoxynucleotide triphosphates (dNTPs) to the double-stranded DNA substrate to yield a first reaction mixture, the first oligonucleotide comprises a third 3’ terminal portion complementary to at least a portion of the first 3’ terminal

portion of the first strand and a 5' phosphate, the third oligonucleotide comprises a first 3' blocking group, the first primer comprising a fourth 3' terminal portion that is complementary to at least a portion of the second 3' terminal portion of the second strand, wherein the first 3' blocking group is not competent for polymerase chain extension, wherein the second oligonucleotide has a 3' portion complementary a 3' portion of the third oligonucleotide and the first oligonucleotide has a 5' portion complementary to a 5' portion of the third oligonucleotide;

(iii) incubating the first reaction mixture under a first set of conditions comprising a ligation temperature for a ligation duration sufficient for the first oligonucleotide and the second oligonucleotide to anneal to the third oligonucleotide in a 3'-5' direction to yield a second reaction mixture comprising the first primer, the double-stranded DNA substrate, and a second primer comprising the first oligonucleotide ligated to the second oligonucleotide;

(iv) subjecting the second reaction mixture to at least 3 PCR cycles, wherein each cycle independently comprises a denaturation temperature for a denaturation duration, an annealing temperature for an annealing duration, and an extension temperature for an extension duration, to yield a third reaction mixture comprising a double-stranded library molecule comprising the second primer at a first 5' end of the double-stranded library molecule and comprising the first primer at a second 5' end of the double-stranded library molecule.

139. The method of claim 138, further comprising:

(v) subjecting the third reaction mixture to additional PCR cycles to amplify the double-stranded library molecule.

140. The method of claim 138, wherein the ligation temperature is less than a melting temperature of the double-stranded DNA substrate.

141. The method of claim 138, wherein the ligation temperature is less than a melting temperature of the first oligonucleotide and the third oligonucleotide, and wherein the ligation temperature is less than a melting temperature of the second oligonucleotide and third oligonucleotide.

142. The method of claim 138, wherein the ligase is a thermolabile ligase capable of ligation in a low magnesium PCR buffer.

143. The method of claim 138, wherein the ligase is T3 DNA ligase.

144. The method of claim 143, wherein the ligase is added at about 30 to about 300 enzyme units per 50 µL of the first reaction mixture.

145. The method of claim 138, wherein the ligase is temperature-sensitive, and wherein the denaturation temperature during a first of the at least 3 PCR cycles in step (iv) is sufficient to inactivate the ligase.

146. The method of claim 138, wherein step (ii) further comprises adding a fourth oligonucleotide and a fifth oligonucleotide, wherein the second oligonucleotide further comprises a 5' phosphate, wherein the fifth oligonucleotide comprises a second 3' blocking group not competent for polymerase chain extension, wherein the fifth oligonucleotide further comprises a 5' portion complementary to a 5' portion of the second oligonucleotide and a 3' portion complementary to a 3' portion of the fourth oligonucleotide, wherein the first set of conditions is further sufficient for the second oligonucleotide and the fourth oligonucleotide to anneal to the fifth oligonucleotide and for the ligase to ligate the fourth oligonucleotide to the second oligonucleotide, thereby yielding the second primer comprising, in a 5' to 3' direction, the fourth oligonucleotide, the second oligonucleotide, and the first oligonucleotide.

147. The method of claim 146, wherein the ligation temperature is less than a melting temperature of the second oligonucleotide and the fifth oligonucleotide, and wherein the ligation temperature is less than a melting temperature of the fourth oligonucleotide and the fifth oligonucleotide.

148. The method of claim 146, wherein the second 3' blocking group is selected from a C3 spacer, hexanediol, Spacer 9, Spacer 18, three or more rU bases, a phosphate, and 2'-O-methyl bases.

149. The method of claim 146, wherein the fourth oligonucleotide has a length of about 5 to about 100 bases.

150. The method of claim 146, wherein the fifth oligonucleotide has a length of about 10 to about 50 bases.

151. The method of claim 146, wherein a melting temperature of the fifth oligonucleotide and the fourth oligonucleotide and second oligonucleotide is lower than the annealing temperature and the extension temperature.

152. The method of any of claims 138-151, wherein steps (i)-(iv) are performed in the same tube.

153. The method of any of claims 138-151, wherein no purification is performed between steps (iii) and (iv).

154. The method of any of claims 138-153, wherein the first oligonucleotide has a length from about 5 to about 100 bases.

155. The method of any of claims 138-154, wherein the second oligonucleotide has a length from about 5 to about 100 bases.

156. The method of any of claims 138-155, wherein the third oligonucleotide has a length from about 10 to about 50 bases.

157. The method of any of claims 138-156, wherein the first 3' blocking group is selected from a C3 spacer, hexanediol, Spacer 9, Spacer 18, three or more rU bases, a phosphate, and 2'-O-methyl bases.

158. The method of any of claims 138-157, wherein the DNA polymerase is selected from the group consisting of Kapa HiFi DNA Polymerase (Roche), NEB Q5 DNA Polymerase (NEB), PrimeStar GXL DNA Polymerase (Takara) and High Fidelity DNA Polymerase (Qiagen).

159. The method of any of claims 138-157, wherein the DNA polymerase is not active at the ligation temperature.

160. The method of claim 159, wherein the DNA polymerase further comprises a hot start antibody or aptamer, and wherein the hot start antibody or aptamer increases an activation temperature of the DNA polymerase.

161. The method of claim 160, wherein the DNA polymerase is selected from the group consisting of Kapa HiFi Hot Start DNA Polymerase (Roche), NEB Q5 Hot Start DNA Polymerase (NEB), PrimeStar GXL Hot Start DNA Polymerase (Takara) and High Fidelity Hot Start DNA Polymerase (Qiagen).

162. The method of any of claims 138-157, wherein the DNA polymerase is a hot start polymerase, and wherein a denaturation temperature during a first of the at least 3 PCR cycles in step (iv) is sufficient to activate the DNA polymerase.

163. The method of any of claims 138-162, wherein the ligation temperature is from about 25 °C to about 40 °C.

164. The method of any of claims 138-163, wherein the denaturation temperature during the at least 3 PCR cycles is independently from about 95 °C to about 98 °C.

165. The method of any of claims 138-164, wherein the annealing temperature during the at least 3 PCR cycles is independently from about 55 ºC to about 65 ºC.

166. The method of any of claims 138-165, wherein the extension temperature during the at least 3 PCR cycles is independently from about 62 º to about 72 ºC.

167. The method of any of claims 138-166, wherein the denaturation duration during the at least 3 PCR cycles is independently about 30 seconds to about 2 minutes.

168. A method for ligation-coupled polymerase chain reaction (PCR), comprising:

(i) providing a double-stranded DNA substrate comprising a first strand and a second strand, the first strand comprising a first 3' terminal portion and a first 5' terminal portion and the second strand comprising a second 3' terminal portion and a second 5' terminal portion;

(ii) adding a ligase, a first oligonucleotide, a second oligonucleotide, a third oligonucleotide, a fourth oligonucleotide, a fifth oligonucleotide, an sixth oligonucleotide, a DNA polymerase and deoxynucleotide triphosphates (dNTPs) to the double-stranded DNA substrate to yield a first reaction mixture, the first oligonucleotide comprises a third 3' terminal portion complementary to at least a portion of the first 3' terminal portion of the first strand and a 5' phosphate, the third oligonucleotide comprises a first 3' blocking group, wherein the second oligonucleotide has a 3' portion complementary a 3' portion of the third oligonucleotide and the first oligonucleotide has a 5' portion complementary to a 5' portion of the third oligonucleotide, the fourth oligonucleotide comprises a fourth 3' terminal portion complementary to at least a portion of the second 3' terminal portion of the second strand and a 5' phosphate, the sixth oligonucleotide comprises a 5' portion complementary to a 5' portion of the fourth oligonucleotide, a 3' portion complementary to a 3' portion of the fifth oligonucleotide, and a second 3' blocking group, wherein the first 3' blocking group and the second 3' blocking group are not competent for polymerase chain extension;

(iii) incubating the first reaction mixture under a first set of conditions comprising a ligation temperature for a ligation duration sufficient for the first oligonucleotide and the second oligonucleotide to anneal to the third oligonucleotide in a 3'-5' direction and for the fourth oligonucleotide and fifth oligonucleotide to anneal to the sixth oligonucleotide in a 3'-5' direction, to yield a second reaction mixture comprising a first primer comprising the fourth oligonucleotide ligated to the fifth oligonucleotide, the double-stranded DNA substrate, and a second primer comprising the first oligonucleotide ligated to the second oligonucleotide;

(iv) subjecting the second reaction mixture to at least 3 PCR cycles, wherein each cycle independently comprises a denaturation temperature for a denaturation duration, an annealing temperature for an annealing duration, and an extension temperature for an extension duration, to yield a third reaction mixture comprising a double-stranded library molecule comprising the second primer at a first 5' end of the double-stranded library molecule and comprising the first primer at a second 5' end of the double-stranded library molecule.

169. The method of claim 168, further comprising:

(v) subjecting the third reaction mixture to additional PCR cycles to amplify the double-stranded library molecule.

170. The method of claim 168, wherein the ligation temperature is less than a melting temperature of the double-stranded DNA substrate.

171. The method of claim 168, wherein the ligation temperature is less than a melting temperature of the first oligonucleotide and the third oligonucleotide, wherein the ligation temperature is less than a melting temperature of the second oligonucleotide and third oligonucleotide, wherein the ligation temperature is less than a melting temperature of the fourth oligonucleotide and sixth oligonucleotide, and wherein the ligation temperature is less than a melting temperature of the fifth oligonucleotide and sixth oligonucleotide.

172. The method of claim 168, wherein the ligase is a thermolabile ligase capable of ligation in a low magnesium PCR buffer.

173. The method of claim 168, wherein the ligase is T3 DNA ligase.

174. The method of claim 173, wherein the ligase is added at about 30 to about 300 enzyme units per 50 µL of the first reaction mixture.

175. The method of claim 168, wherein the ligase is temperature-sensitive, and wherein the denaturation temperature during a first of the at least 3 PCR cycles in step (iv) is sufficient to inactivate the ligase.

176. The method of any of claims 168-175, wherein the first 3' blocking group and the second 3' blocking group are each independently selected from a C3 spacer, hexanediol, Spacer 9, Spacer 18, three or more rU bases, a phosphate, and 2'-O-methyl bases.

177. The method of any of claims 168-176, wherein a melting temperature of the first oligonucleotide and the second oligonucleotide and the third oligonucleotide is lower than the

~~annealing temperature and the extension temperature, and wherein a melting temperature of the fourth oligonucleotide and fifth oligonucleotide and the sixth oligonucleotide is lower than the annealing temperature and the extension temperature.~~

~~178. The method of any of claims 168-177, wherein steps (i) (iv) are performed in the same tube.~~

~~179. The method of any of claims 168-177, wherein no purification is performed between steps (iii) and (iv).~~

~~180. The method of any of claims 138-179, wherein the first oligonucleotide has a length from about 5 to about 100 bases.~~

~~181. The method of any of claims 138-180, wherein the second oligonucleotide has a length from about 5 to about 100 bases.~~

~~182. The method of any of claims 138-181, wherein the third oligonucleotide has a length from about 10 to about 50 bases.~~

~~183. The method of any of claims 138-182, wherein the fourth oligonucleotide has a length from about 5 to about 100 bases.~~

~~184. The method of any of claims 138-183, wherein the fifth oligonucleotide has a length from about 5 to about 100 bases.~~

~~185. The method of any of claims 138-141, wherein the sixth oligonucleotide has a length from about 10 to about 50 bases.~~

~~186. The method of any of claims 138-185, wherein the first 3' blocking group and the second 3' blocking group are each independently selected from the group consisting of a C3 spacer, hexanediol, Spacer 9, Spacer 18, three or more rU bases, a phosphate, and 2'-O-methyl bases.~~

~~187. The method of any of claims 138-186, wherein the DNA polymerase is selected from the group consisting of Kapa HiFi DNA Polymerase (Roche), NEB Q5 DNA Polymerase (NEB), PrimeStar GXL DNA Polymerase (Takara) and High Fidelity DNA Polymerase (Qiagen).~~

~~188. The method of any of claims 138-186, wherein the DNA polymerase is not active at the ligation temperature.~~

~~189. The method of claim 188, wherein the DNA polymerase further comprises a hot start antibody or aptamer, and wherein the hot start antibody or aptamer increases an activation temperature of the DNA polymerase.~~

190. The method of claim 189, wherein the DNA polymerase is selected from the group consisting of Kapa HiFi Hot Start DNA Polymerase (Roche), NEB Q5 Hot Start DNA Polymerase (NEB), PrimeStar GXL Hot Start DNA Polymerase (Takara) and High Fidelity Hot Start DNA Polymerase (Qiagen).

191. The method of any of claims 138-186, wherein the DNA polymerase is a hot start polymerase, and wherein a denaturation temperature during a first of the at least 3 PCR cycles in step (iv) is sufficient to activate the DNA polymerase.

192. The method of any of claims 138-191, wherein the ligation temperature is from about 25 ºC to about 40 ºC.

193. The method of any of claims 138-192, wherein the denaturation temperature during the at least 3 PCR cycles is independently from about 95 ºC to about 98 ºC.

194. The method of any of claims 138-193, wherein the annealing temperature during the at least 3 PCR cycles is independently from about 55 ºC to about 65 ºC.

195. The method of any of claims 138-194, wherein the extension temperature during the at least 3 PCR cycles is independently from about 62 º to about 72 ºC.

196. The method of any of claims 138-195, wherein the denaturation duration during the at least 3 PCR cycles is independently about 30 seconds to about 2 minutes.

197. The method of any of claims 138-196, wherein the denaturing temperature during the first of the at least 3 PCR cycles is greater than a melting temperature of the double-stranded DNA substrate.

198. The method of any of claims 138-197, wherein step (ii) further comprises adding a seventh oligonucleotide and an eighth oligonucleotide, wherein the second oligonucleotide further comprises a 5’ phosphate, wherein the eighth oligonucleotide comprises a third 3’ blocking group not competent for polymerase chain extension, wherein the eighth oligonucleotide further comprises a 3’ portion complementary to a 3’ portion of the seventh oligonucleotide and a 5’ portion complementary to the second oligonucleotide, wherein the first set of conditions is further sufficient for the seventh oligonucleotide and the second oligonucleotide to anneal to the eighth oligonucleotide and for the ligase to ligase the seventh oligonucleotide to the second oligonucleotide, thereby yielding the second primer comprising, in a 5’ to 3’ direction, the seventh oligonucleotide, the second oligonucleotide, and the first oligonucleotide.

199. The method of claim 198, wherein the ligation temperature is less than a melting temperature of the seventh oligonucleotide and second oligonucleotide and eighth oligonucleotide.

200. The method of any of claims 198-199, wherein the annealing temperature and the extension temperature during at least the first of the at least 3 PCR cycles is greater than the melting temperature of the seventh oligonucleotide and second oligonucleotide and eighth oligonucleotide.

201. A kit comprising:

a first indexing primer comprising a 3' terminal portion;

a second indexing primer comprising the 3' terminal portion;

a first ligase; and

a first DNA polymerase.

202. The kit of claim 201, further comprising a blocker oligonucleotide, wherein the blocker oligonucleotide comprises a 5' portion that is at least partially complementary to the 3' terminal portion.

203. The kit of claim 202, wherein the blocker oligonucleotide is pre-annealed to the second indexing primer.

204. The kit of any of claims 202-203, wherein the blocker oligonucleotide comprises a first additional portion 3' of the 5' portion which is complementary to the second indexing primer and not complementary to the first indexing primer.

205. The kit of claim 204, wherein the blocker oligonucleotide further comprises a 3' hydroxyl group and a hairpin portion positioned 3' to the first additional portion, wherein the hairpin portion comprises a first hairpin sequence positioned 5' of a second hairpin sequence, wherein the first hairpin sequence and the second hairpin sequence are complementary and can form a hairpin.

206. The kit of claim 205, wherein the hairpin portion further comprises a third hairpin sequence between the first hairpin sequence and the second hairpin sequences.

207. The kit of claim 206, wherein the third hairpin sequence forms a loop sufficient to allow formation of a stable stem-loop structure with the first and second hairpin sequences.

208. The kit of claim 206, wherein the third hairpin sequence has a length of about 4 bases to about 20 bases.

209. The kit of any of claims 202-204, wherein the blocker oligonucleotide further comprises a second additional portion positioned between the 5' portion and the first additional portion, wherein the second additional portion is not complementary to the first indexing primer, and wherein the second additional portion is not complementary to the second indexing primer.

210. The kit of claim 208, wherein the second additional portion has a length from about 1 to about 30 bases.

211. The kit of any of claims 202-204 and 209-211, wherein the blocker oligonucleotide further comprises a 3' modification to block polymerase extension.

212. The kit of claim 211, wherein the 3' modification to block polymerase extension is selected from the group consisting of a C3 carbon spacer, hexanediol, spacer 9, spacer 18, phosphate, 2',3'-dideoxynucleosides ddA, ddT, ddC and ddG, 3'-deoxynucleosides 3'-A, 3'-T, 3'-C and 3'-G, RNA nucleotides such as rU, 3-O-methyl nucleotides, and a DNA sequence that is not complementary to the second indexing primer.

213. The kit of any of claims 201-212, wherein the first indexing primer and the second indexing primer each independently have a length from about 20 bases to about 100 bases.

214. A kit, comprising:

a first indexing primer comprising a first 3' terminal portion;

a second indexing primer comprising the second 3' terminal portion;

a 5' adapter comprising a third 3' terminal portion;

a first ligase; and

a first DNA polymerase.

215. The kit of claim 214, wherein the 5' adapter further comprises a first 5' portion positioned 5' to the third 3' terminal portion which comprises at least a portion of the first 3' terminal portion of the first indexing primer, a second 5' portion positioned 5' to the first 5' portion and complementary to the first 5' portion, and a replication blocker capable of blocking the DNA polymerase positioned at a 5' end of the first 5' portion.

216. The kit of claim 215, wherein the first 5' portion and the second 5' portion each have a length of about 12 bases to about 20 bases.

~~217. The kit of any of claims 214-216, wherein the 5' adapter further comprises an intervening sequence positioned between the first 5' portion and the second 5' portion.~~

~~218. The kit of claim 217, wherein the intervening sequence has a length of about 4 to about 20 bases.~~

~~219. The kit of any of claims 214-218, wherein the replication block is selected from the group consisting of a stable abasic site, a C3 spacer, hexandiol, Spacer 9, Spacer 18, 3 or more rU bases, and 2'-O-methyl RNA bases.~~

~~220. The kit of any of claims 214-219, wherein the 5' adapter has a length from about 25 bases to about 100 bases.~~

~~221. The kit of any of claims 214-220, wherein the first 5' portion and second 5' portion can form a hairpin.~~

~~222. The kit of any of claims 201-221, wherein the first ligase is a thermolabile ligase capable of ligation in a low magnesium buffer.~~

~~223. The kit of claim 222, wherein the first ligase is T3 DNA ligase.~~

~~224. The kit of any of claims 201-223, wherein the first indexing primer further comprises a 5' tail sequence comprising the sequence of SEQ ID NO: 1, wherein the DNA polymerase has 3'-5' exonuclease activity.~~

~~225. The kit of any of claims 201-224, wherein the second indexing primer further comprises the 5' tail sequence, wherein the DNA polymerase has 3'-5' exonuclease activity.~~

~~226. The kit of any of claims 201-225, wherein the first indexing primer further comprises a 5' tail sequence comprising two or more deoxynucleotides 5' of three or more ribonucleotide bases, wherein the DNA polymerase has 3'-5' exonuclease activity.~~

~~227. The kit of claim 226, wherein the second indexing primer further comprises the 5' tail sequence, wherein the DNA polymerase has 3'-5' exonuclease activity.~~

~~228. The kit of any of claims 224-227, further comprising a probe oligonucleotide complementary to the 5' tail sequence and comprising a modification to provide resistance to digestion by an enzyme with 3' exonuclease activity, the enzyme with 3' exonuclease activity, and a second ligase.~~

~~229. The kit of claim 228, wherein the enzyme with exonuclease activity is Exonuclease III.~~

~~230. The kit of any of claims 201-229, further comprising a target-specific primer pair.~~

~~231. The kit of any of claims 201-229, further comprising a plurality of target-specific primer pairs.~~

~~232. The kit of any of claims 201-231, further comprising a universal primer comprising dU bases, ribonucleotides or inosine bases.~~

~~233. The kit of any of claims 230-232, further comprising a second DNA polymerase.~~

~~234. The kit of claim of any of claims 201-233, further comprising an enzyme capable of cleaving dU bases.~~

~~235. The kit of claim 234, wherein the enzyme capable of cleaving dU bases is a combination of uracil DNA glycosylase and Endonuclease VIII.~~

~~236. The kit of any of claims 201-233, further comprising an enzyme capable of cleaving ribonucleotides.~~

~~237. The kit of claim 236, wherein the enzyme capable of cleaving ribonucleotides is RNase H.~~

~~238. The kit of any of claims 201-233, further comprising an enzyme capable of cleaving inosine bases.~~

~~239. The kit of claim 238, wherein the enzyme capable of cleaving inosine bases is Endonuclease V.~~

~~240. The kit of any of claims 201-239, wherein the first DNA polymerase and second DNA polymerase are each independently selected from the group consisting of Kapa HiFi DNA Polymerase (Roche), NEB Q5 DNA Polymerase (NEB), PrimeStar GXL DNA Polymerase (Takara) and High Fidelity DNA Polymerase (Qiagen).~~

~~241. The kit of any of claims 201-240, wherein the first DNA polymerase further comprises a hot start antibody or aptamer, and wherein the hot start antibody or aptamer increases an activation temperature of the DNA polymerase.~~

~~242. The kit of claim 241, wherein the first DNA polymerase is selected from the group consisting of Kapa HiFi Hot Start DNA Polymerase (Roche), NEB Q5 Hot Start DNA Polymerase (NEB), PrimeStar GXL Hot Start DNA Polymerase (Takara) and High Fidelity Hot Start DNA Polymerase (Qiagen).~~

~~243. The kit of any of claims 215-242, wherein the first indexing primer and the second indexing primer each independently have a length from about 20 bases to about 100 bases.~~

~~244. A method for ligation-coupled polymerase chain reaction (PCR), comprising:~~

(i) providing a partially double-stranded DNA substrate comprising a first strand and a second strand, the partially double-stranded DNA substrate comprising a first 3' overhang, a double-stranded portion, and a second 3' overhang,

the first strand comprising, in a 5' to 3' direction: a first 5' end, a first portion, and a second portion,

the second strand comprising, in a 5' to 3' direction: a second 5' end, a third portion, and a fourth portion of the partially double-stranded DNA substrate,

wherein the first portion of the first strand and the third portion of the second strand are complementary and form the double-stranded portion,

wherein the second portion of the first strand forms the first 3' overhang,

wherein the fourth portion of the second strand forms the second 3' overhang,

wherein the second portion of the first strand and the fourth portion of the second strand each comprise a first common nucleotide sequence positioned at a 5' end of the first 3' overhang and the second 3' overhang, respectively;

(ii) adding a plurality of first indexing primers, a plurality of second indexing primers, a ligase, a DNA polymerase and deoxynucleotide triphosphates (dNTPs) to the partially double-stranded DNA substrate to yield a first reaction mixture, wherein each of the plurality of first indexing primers comprise a first 3' terminal portion complementary to the first common nucleotide sequence, and wherein each of the plurality of second indexing primers comprise a second 3' terminal portion complementary to the first common nucleotide sequence;

(iii) incubating the first reaction mixture under a first set of conditions comprising a ligation temperature for a ligation duration, wherein the first set of conditions is sufficient:

a) for the first 3' terminal portion to anneal to the first common nucleotide sequence, and

b) for the ligase to ligate one of the plurality of first indexing primers to the first 5' end of the first strand and one of the plurality of first indexing primers to the second 5' end of the second strand to yield a second reaction mixture comprising:

a third strand comprising, in a 5' to 3' direction, one of the plurality first indexing primers, the first portion, and the second portion, and

a fourth strand comprising, in a 5' to 3' direction, one of the plurality of first indexing primers, the third portion, and the fourth portion;

(iv) incubating the second reaction mixture under a second set of conditions comprising a first denaturation temperature for a first denaturation duration, a first annealing temperature for a first annealing duration, and a first extension temperature for a first extension duration, wherein the second set of conditions is sufficient:

a) to inactivate the ligase, denature double-stranded DNA, and, optionally, to activate the DNA polymerase,

b) for the second 3' terminal portion of one of the plurality of second indexing primers to anneal to the first common nucleotide sequence of the second portion of the third strand and for the second 3' terminal portion of one of the plurality of second indexing primers to anneal to the first common nucleotide sequence of the fourth portion of the fourth strand, and

c) for the DNA polymerase to extend the one of the plurality of second indexing primers annealed to the first common nucleotide sequence of the second portion of the third strand and for the DNA polymerase to extend the one of the plurality of second indexing primers annealed to the first common nucleotide sequence of the fourth portion of the fourth strand, to yield a third reaction mixture comprising the third strand, the fourth strand, a fifth strand, and a sixth strand, the fifth strand comprising, in a 5' to 3' direction, one of the plurality of second indexing primers, the third portion, and a reverse complement of one of the plurality of first indexing primers, the sixth strand comprising, in a 5' to 3' direction, one of the plurality of second indexing primers, the first portion, and the reverse complement of one of the plurality of first indexing primers;

(v) incubating the third reaction mixture under a third set of conditions comprising a second denaturation temperature for a second denaturation duration, a second annealing temperature for a second annealing duration, and a second extension temperature for a second extension duration, wherein the third set of conditions is sufficient

a) to denature double-stranded DNA,

b) for one of the plurality of first indexing primers to anneal to the reverse complement of one of the plurality of first indexing primers of the fifth strand and for one of the plurality of first indexing primers to anneal to the reverse complement of one of the plurality of first indexing primers of the sixth strand, and

c) for the DNA polymerase to extend the one of the plurality of first indexing primers annealed to the reverse complement of one of the plurality of first indexing primers of the

~~fifth strand and the one of the plurality of first indexing primers annealed to the reverse complement of the one of the plurality of first indexing primers of the sixth strand, to yield a fourth reaction mixture comprising the fifth strand, the sixth strand, a seventh strand, and an eighth strand, the seventh strand comprising, in a 5' to 3' direction, one of the plurality of first indexing primers, the first portion, and a reverse complement of one of the plurality of second indexing primers, the eighth strand comprising, in a 5' to 3' direction, one of the plurality of first indexing primers, the third portion, and the reverse complement of one of the plurality of second indexing primers, wherein the seventh strand is complementary to the fifth strand, and wherein the eighth strand is complementary to the sixth strand; and~~

~~(vi) incubating the fourth reaction mixture under a fourth set of conditions comprising a third denaturation temperature for a third denaturation duration, a third annealing temperature for a third annealing duration, and a third extension temperature for a third extension duration, wherein the fourth set of conditions is sufficient for at least a portion of the plurality of first indexing primers and at least a portion of the plurality of second indexing primers to amplify the fifth strand and seventh strand and the sixth strand and eighth strand.~~

~~245. The method of any of claims 1-118, wherein step (i) is performed during step (iii), further comprising prior to step (ii) providing a starting double-stranded DNA substrate molecule comprising a first starting strand and a second starting strand, wherein the first starting strand and the second starting strand each comprise at least one dU base internal to a 5' end of the first starting strand and the second starting strand respectively, wherein step (ii) further comprises adding an enzyme capable of cleaving the at least one dU base of the double-stranded DNA substrate molecule, wherein the second set of conditions in step (iv) is further sufficient to inactivate the enzyme capable of cleaving the at least one dU base, whereby step an intermediate of step (iii) is the partially double-stranded DNA substrate which can then be ligated according to step (iii).~~

~~246. The method of claim 245, wherein the enzyme capable of cleaving the at least one dU base is a combination of UDG and Endonuclease VIII.~~

~~247. The method of any of claims 1-118, wherein step (i) is performed during step (iii), further comprising prior to step (ii) providing a starting double-stranded DNA substrate molecule comprising a first starting strand and a second starting strand, wherein the first starting strand and the second starting strand each comprise at least one ribonucleotide internal to a 5' end of the first starting strand and the second starting strand respectively, wherein step (ii) further comprises~~

~~adding an enzyme capable of cleaving the at least one ribonucleotide of the double-stranded DNA substrate molecule, wherein the second set of conditions in step (iv) is further sufficient to inactivate the enzyme capable of cleaving the at least one ribonucleotide, whereby step an intermediate of step (iii) is the partially double-stranded DNA substrate which can then be ligated according to step (iii).~~

~~248. The method of claim 247, wherein the enzyme capable of cleaving the at least one ribonucleotide is RNase H.~~

~~249. The method of any of claims 1-118, wherein step (i) is performed during step (iii), further comprising prior to step (ii) providing a starting double-stranded DNA substrate molecule comprising a first starting strand and a second starting strand, wherein the first starting strand and the second starting strand each comprise at least one inosine base internal to a 5' end of the first starting strand and the second starting strand respectively, wherein step (ii) further comprises adding an enzyme capable of cleaving the at least one inosine base of the double-stranded DNA substrate molecule, wherein the second set of conditions in step (iv) is further sufficient to inactivate the enzyme capable of cleaving the at least one inosine base, whereby step an intermediate of step (iii) is the partially double-stranded DNA substrate which can then be ligated according to step (iii).~~

~~250. The method of claim 249, wherein the enzyme capable of cleaving the at least one inosine base is Endonuclease V.~~

~~251. The method of any of claims 245-250, wherein the starting double-stranded DNA substrate molecule comprise complementary strands annealed to 3' overhangs of the starting double-stranded DNA substrate molecule.~~

~~252. The method of claim 252, wherein the complementary strands comprise the dU base, ribonucleotides or inosine bases.~~