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; and

wherein steps (i) through (vi) are performed in a single closed tube.

[13] Novel methods designed to increase library yields and overcome the requirement for qPCR quantification when using the Swift Amplicon multiplexed PCR targeting sequencing method referenced above and other methods are disclosed herein. The methods use indexing primers in a ligation-coupled PCR reaction. There are two methods that each utilize one of two different indexing primer designs that are commonly used in the art: indexing primers that comprise all sequences unique to each adapter but which omit the sequence common to both adapters at their 3' terminus, and indexing primers that comprise the complete, full-length sequence of each adapter, including the sequence common to both adapters at their 3' terminus. The shorter design reduces oligonucleotide synthesis costs while the longer design has a higher PCR efficiency as it can disrupt the secondary structure formed by the common adapter sequence and its reverse complement when priming denatured library molecules.

[14] For either method, the Swift multiplexed PCR step can be performed as previously disclosed without any modification and is summarized below for reference, utilizing a plurality of target-specific primer pairs each comprising a 5' tail sequence comprising a universal adapter sequence, and a universal primer complementary to the universal adapter sequence, where the universal primer contains a modification making it susceptible to cleavage by an endonuclease which is required for the subsequent adapter ligation step. The PCR utilizes a high fidelity proofreading polymerase that is tolerant of the modified base, where the first PCR cycles have elongated annealing times to allow the high complexity of target-specific primer pairs, each of which is at a low concentration, to create universal adapter tagged amplicons from their target sequences. Following the multiplex cycles (2 or more), PCR is continued with shorter annealing times for a second phase of amplification using the universal primer that anneals to the universal

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; and

wherein steps (i) through (vi) are performed in a single closed tube.

2. The method of claim 1, wherein the partially double-stranded DNA substrate has a length of 24 bases to 6000 bases; wherein the first portion and the third portion of the first strand and the second strand, respectively, each have a length of 20 bases to 6000 bases; wherein the second portion of the first strand and the fourth portion of the second strand each have a length of 4 bases

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

3. 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, the first 3' terminal portion, and the second 3' terminal portion have a T_m less than the first annealing temperature.

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

5. The method of claim 1, wherein the ligase is T3 DNA ligase.

6. The method of claim 1, wherein the DNA polymerase is a thermostable DNA polymerase with 3'-5' exonuclease proofreading activity.

7. 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.

8. The method of claim 1, wherein the first denaturation temperature, the second denaturation temperature, and the third denaturation temperature are each independently 95 ^oC to 98 ^oC; and wherein the first denaturation duration, the second denaturation duration, and the third denaturation duration are each independently from 30 seconds to 2 minutes.

9. The method of claim 1, wherein the first annealing temperature, the second annealing temperature, and the third annealing temperature are each independently 55 ^oC to 65 ^oC; and wherein the first annealing duration, the second annealing duration, and the third annealing duration are each independently 10 seconds to 60 seconds.

10. The method of claim 1, wherein the first extension temperature, the second extension temperature, and the third extension temperature are each independently 62 ^oC to 72 ^oC; and

wherein the first extension duration, the second extension duration, and the third extension duration are each independently 30 seconds to 5 minutes.

11. 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 100 nM to 1 μM.

12. 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.

13. 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 form a first double-stranded product having a first 5' overhang and a second 5' overhang, and whereby the sixth strand and eighth strand 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' exonuclease 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.

14. The method of claim 13, further comprising sequencing the normalized NGS library.