

Claims

1. A method of nucleic acid manipulation comprising:

5 (i) hybridizing a double stranded primer to the 3' end of a single stranded nucleic acid template, wherein said primer comprises a double stranded region and a single stranded region, wherein the single stranded region is a 3' overhang region and wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of the nucleic acid template, wherein said single stranded 3' overhang region comprises a degenerate sequence or a sequence
10 comprising universal bases, and wherein the double stranded primer is made up of two separate strands;

(ii) at least one round of polymerization using a polypeptide with 5' to 3' DNA polymerization activity, wherein at least the first round of polymerization comprises using a polypeptide with 5' to 3' DNA polymerization activity to carry out a primer
15 extension reaction to synthesise nucleotides in a template dependent manner from the 3' end of the single stranded region of said hybridized double stranded primer;

wherein the hybridizing step (i) and at least the first primer extension reaction from the 3' end of the single stranded region of said hybridized double stranded primer in step (ii) takes place without the formation of a phosphodiester
20 bond between the 3' end of the nucleic acid template and the double stranded primer of step (i).

2. A method of nucleic acid amplification comprising:

25 (i) hybridizing a double stranded primer to the 3' end of a single stranded nucleic acid template, wherein said primer comprises a double stranded region and a single stranded region, wherein the single stranded region is a 3' overhang region and wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of the nucleic acid template, wherein said single stranded 3' overhang region comprises a degenerate sequence or a sequence
30 comprising universal bases, and wherein the double stranded primer is made up of two separate strands;

(ii) at least one round of polymerization using a polypeptide with 5' to 3' DNA polymerization activity in order to form an amplification template, wherein at least the first round of polymerization comprises using a polypeptide with 5' to 3' DNA
35 polymerization activity to carry out a primer extension reaction to synthesise

- 134 -

nucleotides in a template dependent manner from the 3' end of the single stranded region of said hybridized double stranded primer; and

(iii) at least one amplification step to form an amplified nucleic acid product;

wherein the hybridizing step (i) and at least the first primer extension

5 reaction from the 3' end of the single stranded region of said hybridized double stranded primer in step (ii) takes place without the formation of a phosphodiester bond between the 3' end of the nucleic acid template and the double stranded primer of step (i).

10 3. The method of claim 1 or claim 2, wherein said double stranded region of said double stranded primer comprises a nucleic acid fragment to be joined to said nucleic acid template, a primer binding site, a control region, a promoter, a terminator, an operator, a repressor, an enhancer, a silencer, an insulator or other response element, a sequencing adaptor region or other element permitting or
15 facilitating sequencing, a restriction site, a label, an identifier sequence, a barcode, a forensic signature, or combinations thereof.

4. The method of any one of claims 1 to 3, wherein said double stranded primer comprises a further single stranded 3' overhang region, and preferably wherein said
20 double stranded region of said double stranded primer comprises one or more of a nucleic acid fragment to be joined to said nucleic acid template, a primer binding site, a control region, a promoter, a terminator, an operator, a repressor, an enhancer, a silencer, an insulator or other response element, a sequencing adaptor region or other element permitting or facilitating sequencing, a restriction site, a
25 label, an identifier sequence, a barcode, or a forensic signature, which may be the same or different, or combinations thereof.

5. The method of any one of claims 1 to 4, wherein said single stranded 3' overhang region comprises universal bases selected from the group consisting of
30 inosine, 2-amino purine, diaminopurine, 5-nitroindole, 5-methyl isodeoxycytosine, iso deoxyguanine, or a mixture thereof.

6. The method of any one of claims 1 to 5, wherein said single stranded 3' overhang region is up to 6 or 12 base pairs in length.

35

7. The method of any one of claims 1 to 6 wherein a single type of double stranded primer is used.

5 8. The method of any one of claims 1 to 7 wherein two or more different types of double stranded primers are used.

10 9. The method of claim 8, wherein the two or more different types of double stranded primer contain different primer binding sites, or wherein one double stranded primer contains a promoter region and the other a terminator region, or wherein one double stranded primer contains a promoter region and the other a primer binding site.

15 10. The method of any one of claims 1 to 9, wherein one or more of the double stranded primers is a hybrid double stranded primer wherein one strand comprises DNA and the other strand comprises RNA.

11. The method of any one of claims 1 to 10, wherein said method further comprises the use of a lock primer which cannot be subjected to chain elongation.

20 12. The method of any one of claims 1 to 11, wherein said method is carried out under isothermal conditions.

25 13. The method of any one of claims 1 to 12, wherein said method is used in sequencing applications, nucleic acid labelling, joining nucleic acid fragments together, or for incorporation of primer sites, promoter sites, terminator sites and/or restriction sites.

30 14. The method of any one of claims 1 to 13, wherein one or more strands of one or more of the double stranded primers comprises a chimeric RNA/DNA or DNA/RNA sequence.

15. The method of any one of claims 1 to 14, wherein the double stranded primer is immobilized to a solid phase before step (i) is carried out.

16. The method of claim 15, wherein the solid phase is a nanoparticle, preferably a magnetic nanoparticle.

5 17. A method of nucleic acid manipulation comprising a hybridizing step wherein a double stranded primer is hybridized to the 3' end of a single stranded nucleic acid template, wherein said primer comprises a double stranded region and a single stranded region, wherein the single stranded region is a 3' overhang region and wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of the nucleic acid template, wherein said single stranded 3'
10 overhang region comprises a degenerate sequence or a sequence comprising universal bases, and wherein the double stranded primer is made up of two separate strands;

wherein said hybridizing step takes place without the formation of a phosphodiester bond between the 3' end of the nucleic acid template and the
15 double stranded primer of the hybridizing step.

18. A method of nucleic acid manipulation comprising a hybridizing step wherein a double stranded primer is hybridised to the 5' end of a single stranded nucleic acid template, wherein said primer comprises a double stranded region and a single
20 stranded region, wherein the single stranded region is a 5' overhang region and wherein the 5' overhang region enables the double stranded primer to target and hybridize to the 5' end of the nucleic acid template, wherein said single stranded 5' overhang region comprises a degenerate sequence or a sequence comprising universal bases, and wherein the double stranded primer is made up of two
25 separate strands;

wherein said hybridizing step takes place without the formation of a phosphodiester bond between the 5' end of the nucleic acid template and the double stranded primer of the hybridising step.

30 19. A method of Systematic Evolution of Ligands by Exponential Enrichment (SELEX) comprising:

(a) contacting a library of single stranded nucleic acid molecules with a ligand of interest, wherein said library is a library of truncated nucleic acid molecules in which the 3' ends of the nucleic acid molecules do not have a 3'OH
35 group;

- 137 -

(b) selecting for nucleic acid molecules which bind said ligand;

(c) use of a double stranded primer to amplify the nucleic acid molecules which bind to said ligand, wherein said double stranded primer hybridizes to the 3' end of the single stranded molecules selected in step (b), wherein said primer comprises a double stranded region and a single stranded region, wherein the single stranded region is a 3' overhang region and wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of the molecules selected in step (b), wherein said single stranded 3' overhang region comprises a degenerate sequence or a sequence comprising universal bases, and wherein the double stranded primer is made up of two separate strands; and optionally

(d) use said amplified nucleic acid molecules as a new library of nucleic acid molecules to repeat steps (a) to (c) one or more times.

20. The method of claim 19, wherein the libraries of step (a) are produced using a polypeptide with RNA or DNA polymerase activity in the presence of chain terminators.

21. The method of claim 19 or claim 20, wherein said amplification of nucleic acid molecules in step (c) is carried out in accordance with any one of claims 2 to 16.

22. The method of any one of claims 19 to 21, wherein said library of step (a) contains nucleic acid molecules of a predetermined fixed length and nucleic acid molecules with decreasing lengths from said predetermined fixed length.

23. The method of any one of claims 19 to 22, wherein the amplification products produced in step (c) are nucleic acid molecules which do not contain additional oligonucleotide regions, such as constant or fixed primer binding sites, promoter, terminator, adaptor regions or other element permitting or facilitating function as a primer binding site, at the 5' or 3' end of the molecule, or only contain such additional oligonucleotide regions at the 5' end of the molecule.

24. The method of any one of claims 19 to 23, wherein said method step (c) is carried out without the formation of a phosphodiester bond between the 3' end of the nucleic acid molecules selected in step (b) and the double stranded primer.

25. The method of any one of claims 19 to 24, wherein a single stranded nucleic acid molecule which can specifically bind to said ligand is identified.

5 26. A solid phase comprising immobilized double stranded primers, wherein said double stranded primers comprise a double stranded region and a single stranded region, wherein the single stranded region is a 3' overhang region and wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of a single stranded nucleic acid template, wherein said single stranded
10 3' overhang region comprises a degenerate sequence or a sequence comprising universal bases, and wherein the double stranded primer is made up of two separate strands.

27. The solid phase of claim 26, wherein said double stranded primers are
15 immobilized to the solid phase through either the 5' end of the strand containing the 3' overhang region or the 3' end of the non-overhang strand.

28. The solid phase of claim 26 or claim 27, wherein said solid phase is a
20 nanoparticle, preferably a magnetic nanoparticle.

29. A method of preparing a library, comprising the following steps:
(A) hybridizing single stranded nucleic acid templates to the solid phase of claim 27
or claim 28;
(B) polymerization using a polypeptide with 5' to 3' DNA polymerization activity to
25 carry out a primer extension reaction to synthesize nucleotides in a template dependent manner from the 3' end of the single stranded region of the dsPrimer;
(C) removing the non-immobilized strands from the solid phase, wherein said non-immobilized strands form a library of single stranded molecules; optionally
(D) retaining the immobilized single stranded molecules as a backup library.

30 30. The method of claim 29 comprising one or more of the following additional steps:
(E) after the hybridization step (A) and before step (C) carrying out a step in which the gap between the hybridized single stranded nucleic acid molecules and the 5'

end of the strand of the dsPrimer which does not contain the 3' overhang region is repaired;

(F) after the polymerization step (B) and before step (C) ligating a double stranded adaptor molecule to the free ends of the immobilized double stranded polymerized product which are not in contact with the solid phase.

31. A method of preparing a library, comprising the following steps:

(A) hybridizing single stranded nucleic acid templates, wherein said templates comprise a known primer sequence at the 5' end, to the solid phase of claim 27 or claim 28, wherein the double stranded primers of said solid phase are immobilized to the solid phase through the 5' end of the strand containing the 3' overhang region;

(B) polymerization using a polypeptide with 5' to 3' DNA polymerization activity to carry out a primer extension reaction to synthesize nucleotides in a template dependent manner from the 3' end of the single stranded region of the dsPrimer;

(C) removing the non-immobilized strands containing the nucleic acid template molecules from the solid phase;

(D) resynthesizing the nucleic acid template molecules using a single stranded primer which hybridizes to the complement of the known primer site which has been incorporated at the 3' end of the polymerized strand generated in step (B), hybridizing said primer, after which an enzyme with 5' to 3' DNA polymerization activity is used to carry out a primer extension reaction to synthesize nucleotides in a template dependent manner from the 3' end of said single stranded primer;

(E) removing the non-immobilized strands from the solid phase, wherein said non-immobilized strands form a library of single stranded molecules; optionally

(F) retaining the immobilized single stranded molecules as a backup library.

32. The method of claim 31, wherein the single stranded nucleic acid templates in step (A) are candidate SELEX aptamers, preferably truncated SELEX aptamers.

33. A Library or backup library produced by the methods of any one of claims 29 to 32.

34. A double stranded primer which comprises a double stranded region and two single stranded regions, wherein the single stranded regions are 3' overhang

regions which are present on the 3' end of each strand of the double stranded primer, wherein said single stranded 3' overhang regions comprise a degenerate sequence or a sequence comprising universal bases, and wherein the double stranded primer is made up of two separate strands.

5

35. The double stranded primer of claim 34 wherein said double stranded region of said double stranded primer comprises a nucleic acid fragment to be joined to said nucleic acid template, a primer binding site, a control region, a promoter, a terminator, an operator, a repressor, an enhancer, a silencer, an insulator or other response element, a sequencing adaptor region or other element permitting or facilitating sequencing, a restriction site, a label, an identifier sequence a barcode or a forensic signature, or combinations thereof.

10

36. The double stranded primer of any one of claims 34 or 35, wherein said single stranded 3' overhang region comprises universal bases selected from the group consisting of inosine, 2-amino purine, diaminopurine, 5-nitroindole, 5-methyl isodeoxycytosine, iso deoxyguanine, or a mixture thereof.

15

37. The double stranded primer of any one of claims 34 to 36, wherein said single stranded 3' overhang region is up to 6 or 12 base pairs in length.

20

38. The double stranded primer of any one of claims 34 to 37, wherein the double stranded primers is a hybrid double stranded primer wherein one strand comprises DNA and the other strand comprises RNA.

25

39. The double stranded primer of any one of claims 34 to 38, wherein the double stranded primer is a lock primer which cannot be subjected to chain elongation.

40. The double stranded primer of any one of claims 34 to 39, wherein one or more strands of the double stranded primer comprises a chimeric RNA/DNA or DNA/RNA sequence

30

41. A double stranded primer comprising a double stranded region and a single stranded region, wherein the single stranded region is a 3' overhang region comprising a degenerate sequence or a sequence comprising universal bases,

35

wherein when only one of the strands has a 3' overhang region then the strand which does not contain the 3' overhang region does not have a 5'- PO₃ group, and wherein when both of the strands have a 3' overhang region then one or both of the strands does not have a 5'- PO₃ group.

5

42. The double stranded primer of claim 41 wherein said lack of a 5'- PO₃ group prevents incorporation of said strand which lacks the 5'- PO₃ group into a nucleic acid template primed by the primer.

10

43. A composition comprising the double stranded primer of claim 41 or claim 42 and a nucleic acid template wherein the double stranded primer is hybridized to the 3' end of the nucleic acid template but the strand which lacks the 5'- PO₃ group is not incorporated into the nucleic acid template with a covalent bond and may be removed by heat denaturation, strand displacement or nick translation.

15

44. The double stranded primer of any one of claims 41 to 43 wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of a nucleic acid template.

20

45. The double stranded primer of any one of claims 41 to 44, wherein the double stranded region of said double stranded primer comprises: a nucleic acid fragment to be joined to said nucleic acid template, a primer binding site, a control region, a promoter, a terminator, an operator, a repressor, an enhancer, a silencer, an insulator or other response element, a sequencing adaptor region or other element permitting or facilitating sequencing, a restriction site, a label, an identifier sequence, a barcode, a forensic signature, or combinations thereof.

25

46. A kit or composition comprising the solid phase of any one of claims 26 to 28 or the double stranded primer of any one of claims 34 to 42 or 44 to 45

30

47. A kit or composition comprising:

(i) a double stranded primer which comprises a double stranded region and a single stranded region, wherein the single stranded region is a 3' overhang region and wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of a single stranded nucleic acid template, wherein said

35

single stranded 3' overhang region comprises a degenerate sequence or a sequence comprising universal bases, wherein the double stranded primer is made up of two separate strands, and wherein said double stranded region of said double stranded primer comprises a promoter region; and

- 5 (ii) a double stranded primer which comprises a double stranded region and a single stranded region, wherein the single stranded region is a 3' overhang region and wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of a single stranded nucleic acid template, wherein said single stranded 3' overhang region comprises a degenerate sequence or a
- 10 sequence comprising universal bases, wherein the double stranded primer is made up of two separate strands, and wherein said double stranded region of said double stranded primer comprises a terminator region or a primer binding site.

48. A kit or composition comprising:

- 15 (i) a single type of double stranded primer wherein said double stranded primer comprises a double stranded region and a single stranded region, wherein the single stranded region is a 3' overhang region and wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of a single stranded nucleic acid template, wherein said single stranded 3' overhang region
- 20 comprises a degenerate sequence or a sequence comprising universal bases, wherein the double stranded primer is made up of two separate strands and wherein said double stranded region of said double stranded primer comprises a primer binding site; and, optionally
- (ii) a single type of single stranded primer which can hybridise to said primer
- 25 binding site.

49. A SELEX kit or composition comprising:

- (i) a double stranded primer which comprises a double stranded region and a single stranded region, wherein the single stranded region is a 3' overhang region and
- 30 wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of a single stranded nucleic acid template, wherein said single stranded 3' overhang region comprises a degenerate sequence or a sequence comprising universal bases, wherein the double stranded primer is made up of two separate strands; and

- 143 -

(ii) a library of single stranded nucleic acid aptamers, wherein said library is a library of truncated nucleic acid molecules in which the 3' ends of the nucleic acid molecules do not have a 3'OH group.

- 5 50. The solid phase of any one of claims 26 to 28 or the double stranded primer of any one of claims 34 to 42 or 44 to 45 for use in sequencing applications, nucleic acid labelling, joining nucleic acid fragments together, or for incorporation of primer sites, terminator sites and/or restriction sites.

CLAIMS

1. A method of nucleic acid manipulation comprising:

(i) hybridizing a double stranded primer to the 3' end of a single stranded nucleic acid template, wherein said primer comprises a double stranded region and a single stranded region, wherein the single stranded region is a 3' overhang region and wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of the nucleic acid template, wherein said single stranded 3' overhang region comprises a sequence complementary to the 3' end of the nucleic acid template or a sequence comprising universal bases, and wherein the double stranded primer is made up of two separate strands;

(ii) at least one round of polymerization using a polypeptide with 5' to 3' DNA polymerization activity, wherein at least the first round of polymerization comprises using a polypeptide with 5' to 3' DNA polymerization activity to carry out a primer extension reaction to synthesise nucleotides in a template dependent manner from the 3' end of the single stranded region of said hybridized double stranded primer;

wherein the hybridizing step (i) and at least the first primer extension reaction from the 3' end of the single stranded region of said hybridized double stranded primer in step (ii) takes place without the formation of a phosphodiester bond between the 3' end of the nucleic acid template and the double stranded primer of step (i).

2. The method of claim 1, wherein the sequence complementary to the 3' end of the nucleic acid template comprises a specific defined sequence.

3. The method of either claim 1 or 2, wherein the double stranded primer is immobilized to a solid phase before step (i) is carried out.

4. The method of claim 3, wherein the solid phase is a nanoparticle, preferably a magnetic nanoparticle.

5. A solid phase comprising immobilized double stranded primers, wherein said double stranded primers comprise a double stranded region and a single stranded region, wherein the single stranded region is a 3' overhang region and wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of a single stranded nucleic acid template, wherein said single stranded 3' overhang region

comprises a sequence complementary to the 3' end of the nucleic acid template or a sequence comprising universal bases, and wherein the double stranded primer is made up of two separate strands.

6. The solid phase of claim 5, wherein the sequence complementary to the 3' end of the nucleic acid template comprises a specific defined sequence.

7. The solid phase of either claim 5 or 6, wherein said double stranded primers are immobilized to the solid phase through either the 5' end of the strand containing the 3' overhang region or the 3' end of the non-overhang strand.

8. The solid phase of any one of claims 5 to 7, wherein said solid phase is a nanoparticle, preferably a magnetic nanoparticle.

9. A method of preparing a library, comprising the following steps:

(A) hybridizing single stranded nucleic acid templates to the solid phase of claim 7 or claim 8;

(B) polymerization using a polypeptide with 5' to 3' DNA polymerization activity to carry out a primer extension reaction to synthesize nucleotides in a template dependent manner from the 3' end of the single stranded region of the dsPrimer;

(C) removing the non-immobilized strands from the solid phase, wherein said non-immobilized strands form a library of single stranded molecules; optionally

(D) retaining the immobilized single stranded molecules as a backup library; wherein the hybridizing step (A) and at least the first primer extension reaction from the 3' end of the single stranded region of the hybridized double stranded primer in step (B) takes place without the formation of a phosphodiester bond between the 3' end of the nucleic acid template and the double stranded primer.

10. The method of claim 9 comprising the following additional step:

(E) after the polymerization step (B) and before step (C) ligating a double stranded adaptor molecule to the free ends of the immobilized double stranded polymerized product which are not in contact with the solid phase.

11. A method of preparing a library, comprising the following steps:

- (A) hybridizing single stranded nucleic acid templates, wherein said templates comprise a known primer sequence at the 5' end, to the solid phase of claim 7 or claim 8, wherein the double stranded primers of said solid phase are immobilized to the solid phase through the 5' end of the strand containing the 3' overhang region;
- (B) polymerization using a polypeptide with 5' to 3' DNA polymerization activity to carry out a primer extension reaction to synthesize nucleotides in a template dependent manner from the 3' end of the single stranded region of the dsPrimer;
- (C) removing the non-immobilized strands containing the nucleic acid template molecules from the solid phase;
- (D) resynthesizing the nucleic acid template molecules using a single stranded primer which hybridizes to the complement of the known primer site which has been incorporated at the 3' end of the polymerized strand generated in step (B), hybridizing said primer, after which an enzyme with 5' to 3' DNA polymerization activity is used to carry out a primer extension reaction to synthesize nucleotides in a template dependent manner from the 3' end of said single stranded primer;
- (E) removing the non-immobilized strands from the solid phase, wherein said non-immobilized strands form a library of single stranded molecules; optionally
- (F) retaining the immobilized single stranded molecules as a backup library; wherein the hybridizing step (A) and at least the first primer extension reaction from the 3' end of the single stranded region of the hybridized double stranded primer in step (B) takes place without the formation of a phosphodiester bond between the 3' end of the nucleic acid template and the double stranded primers of the solid phase.

12. A Library or backup library produced by the methods of any one of claims 9 to 11.

13. A double stranded primer which comprises a double stranded region and two single stranded regions, wherein the single stranded regions are 3' overhang regions which are present on the 3' end of each strand of the double stranded primer, wherein said single stranded 3' overhang regions comprise a sequence complementary to the 3' end of a nucleic acid template or a sequence comprising universal bases, and wherein the double stranded primer is made up of two separate strands.

14. A kit comprising:

- (i) a single type of double stranded primer wherein said double stranded primer comprises a double stranded region and a single stranded region, wherein the single stranded region

is a 3' overhang region and wherein the 3' overhang region enables the double stranded primer to target and hybridize to the 3' end of a single stranded nucleic acid template, wherein said single stranded 3' overhang region comprises a sequence complementary to the 3' end of the nucleic acid template or a sequence comprising universal bases, wherein the double stranded primer is made up of two separate strands and wherein said double stranded region of said double stranded primer comprises a primer binding site; and, optionally

(ii) a single type of single stranded primer which can hybridise to said primer binding site.

15. The double stranded primer of claim 13 or the kit of claim 14, wherein the sequence complementary to the 3' end of the nucleic acid template comprises a specific defined sequence.