

CLAIMS

1. A method for determining the sequence bias of a sequencing technique, the method comprising:

- (a) providing a degenerate library of nucleic acid molecules;
- 5 (b) determining the sequence of the nucleic acid molecules in the degenerate library of nucleic acid molecules provided in (a) using the sequencing technique; and
- (c) analysing the sequencing results to determine over- or under-representation of particular nucleic acid molecules.

2. The method according to claim 1, wherein the sequencing technique involves ligation of adaptor molecules to the 5', 3' or 5' and 3' ends of the nucleic acid molecules in the degenerate library of nucleic acid molecules.

3. The method according to claim 2, comprising:

- (a) providing a degenerate library of nucleic acid molecules;
- (b) ligating an oligonucleotide of known sequence with a blocked 3' end (3' adaptor molecule) to the 3' ends of the nucleic acid molecules using a ligase;
- 20 (c) ligating an oligonucleotide of known sequence with a blocked 5' end (5' adaptor molecule) to the 5' ends of the nucleic acid molecules from step (b) using a ligase;
- (d) sequencing the resulting library of ligated nucleic acid molecules; and
- (e) analysing the sequencing results to determine over- or under-representation of particular nucleic acid molecules;

wherein steps (b) and (c) may be carried out in any order.

4. The method according to claim 2, comprising:

- (a) providing a degenerate library of nucleic acid molecules, wherein the nucleic acid molecules have a consistent 3' region and a blocked 3' end;

(b) ligating an oligonucleotide of known sequence with a blocked 5' end (5' adaptor molecule) to the 5' ends of the nucleic acid molecules from step (a) using a ligase;
(c) sequencing the resulting amplified library of nucleic acid molecules;
5 and
(d) analysing the sequencing results to determine over- or under-representation of particular nucleic acid molecules.

5. The method according to claim 2, comprising:

10 (a) providing a degenerate library of nucleic acid molecules, wherein the nucleic acid molecules have a consistent 5' region and a blocked 5' end;
(b) ligating an oligonucleotide of known sequence with a blocked 3' end (3' adaptor molecule) to the 3' ends of the nucleic acid molecules from step (a) using a ligase;
15 (c) sequencing the resulting amplified library of nucleic acid molecules;
and
(d) analysing the sequencing results to determine over- or under-representation of particular nucleic acid molecules.

20 6. The method according to claim 1, comprising:

(a) providing a degenerate library of nucleic acid molecules, wherein the nucleic acid molecules have a consistent 5' region and a consistent 3' region;
(b) sequencing the resulting amplified library of nucleic acid molecules;
25 and
(c) analysing the sequencing results to determine over- or under-representation of particular nucleic acid molecules.

7. The method according to claim 1, comprising:

30 (a) providing a degenerate library of RNA molecules;
(b) ligating an oligonucleotide of known sequence with a blocked 3' end (3' adaptor molecule) to the 3' ends of the RNA molecules using a ligase;

(c) ligating an oligonucleotide of known sequence with a blocked 5' end (5' adaptor molecule) to the 5' ends of the RNA molecules from step (b) using a ligase;

(d) reverse transcribing the RNA molecules into cDNA using a reverse transcriptase and a primer capable of hybridising to the 3' adaptor molecule;

(e) if necessary, creating an amplified library of nucleic acid molecules by PCR of the cDNA molecules obtained in step (d) using a primer capable of hybridising to the complement of the 5' adaptor molecule and a primer capable of hybridising to the 3' adaptor molecule;

(d) sequencing the resulting library of nucleic acid molecules; and

(e) analysing the sequencing results to determine over- or under-representation of particular nucleic acid molecules;

wherein steps (b) and (c) may be carried out in any order.

8. The method according to claim 1, comprising:

(a) providing a degenerate library of DNA molecules;

(b) ligating an oligonucleotide of known sequence with a blocked 3' end (3' adaptor molecule) to the 3' ends of the DNA molecules using a ligase;

(c) ligating an oligonucleotide of known sequence with a blocked 5' end (5' adaptor molecule) to the 5' ends of the DNA molecules from step (b) using a ligase;

(d) amplifying the ligated DNA molecules by asymmetric PCR;

(e) if necessary, creating an amplified library of nucleic acid molecules by PCR of the DNA molecules obtained in step (d) using a primer capable of hybridising to the complement of the 5' adaptor molecule and a primer capable of hybridising to the 3' adaptor molecule;

(d) sequencing the resulting library of nucleic acid molecules; and

(e) analysing the sequencing results to determine over- or under-representation of particular nucleic acid molecules;

wherein steps (b) and (c) may be carried out in any order.

9. A method of reducing the sequence bias of a sequencing technique involving adaptor ligation, the method comprising:

- 5 (a) providing a set of oligonucleotides of known sequence with blocked 3' ends (3' adaptor molecules) and a set of oligonucleotides of known sequence with blocked 5' ends (5' adaptor molecules), wherein the 3' and 5' adaptor molecules comprise one or more degenerate nucleotides;
- (b) ligating the 3' adaptor molecules to the 3' ends of the target nucleic acid molecules using a ligase;
- 10 (c) ligating the 5' adaptor molecules to the 5' ends of the target nucleic acid molecules from step (b) using a ligase; and
- (d) determining the sequence of the target nucleic acid molecules obtained in step (c) using a primer capable of hybridising to the complement of the 5' adaptor molecule and a primer capable of hybridising to the 3' adaptor molecule.

15

10. A method of preferentially detecting a target nucleic acid molecule in a library of nucleic acid molecules, the method comprising:

- 20 (a) providing a set of oligonucleotides of known sequence with blocked 3' ends (3' adaptor molecules) and a set of oligonucleotides of known sequence with blocked 5' ends (5' adaptor molecules), wherein the 3' and 5' adaptor molecules comprise one or more degenerate nucleotides and are preferentially ligatable to the target nucleic acid molecule;
- (b) ligating the 3' adaptor molecule to the 3' ends of the nucleic acid molecules in the library of nucleic acid molecules using a ligase;
- 25 (c) ligating the 5' adaptor molecule to the 5' ends of nucleic acid molecules from step (b) using a ligase;
- (d) creating an amplified library of nucleic acid molecules by PCR of the nucleic acid molecules obtained in step (c) using a primer capable of hybridising to the complement of the 5' adaptor molecule and a primer capable of hybridising to the 3' adaptor molecule;
- 30 (e) sequencing the resulting amplified library of nucleic acid molecules; and

(f) analysing the sequencing results to determine whether the target nucleic acid molecule is present in the library of nucleic acid molecules.

11. A method of preferentially detecting a target nucleic acid molecule in a library
5 of nucleic acid molecules, the method comprising:

(a) providing an oligonucleotide of known sequence (adaptor molecule),
comprising one or more degenerate nucleotides, wherein said
oligonucleotide is preferentially ligatable to the target nucleic acid
molecule;

10 (b) ligating the adaptor molecule to the nucleic acid molecules in the library
using a ligase;

(c) performing quantitative PCR on the molecules obtained in step (b)
using one primer specific for the adaptor molecule and one primer specific
for the target nucleic acid.

15

12. The method according to either claim 10 or claim 11, wherein the target
nucleic acid is associated with a disease or pre-disease state.

13. The method according to either claim 10 or claim 11, wherein the target
20 nucleic acid is associated with a particular organism.

14. The method according to either claim 10 or claim 11, wherein the target
nucleic acid is associated with a particular tissue type.

25 15. The method according to either claim 10 or claim 11, wherein the target
nucleic acid is associated with a particular developmental stage.

16. The method according to any one of claims 1 to 6 or 9 to 15, wherein the
nucleic acid molecules are RNA molecules.

30

17. The method according to any one of claims 1 to 6 or 9 to 15, wherein the
nucleic acid molecules are DNA molecules.

18. A method of generating a cDNA library from a library of RNA molecules, the method comprising:

- 5 (a) providing a set of oligonucleotides of known sequence with blocked 3' ends (3' adaptor molecules) and a set of oligonucleotides of known sequence with blocked 5' ends (5' adaptor molecules), wherein the 3' and 5' adaptor molecules comprise one or more degenerate nucleotides;
- (b) ligating the 3' adaptor molecules to the 3' ends of the RNA molecules using a ligase;
- 10 (c) ligating the 5' adaptor molecules to the 5' ends of the RNA molecules from step (b) using a ligase;
- (d) creating hybrid RNA/DNA template molecules from the RNA molecules obtained in step (c) using a reverse transcriptase enzyme and a primer capable of hybridising to the 3' adaptor molecule; and
- 15 (e) creating a cDNA library by PCR of the hybrid RNA/DNA template molecules obtained in step (d) using a primer capable of hybridising to the complement of the 5' adaptor molecule and a primer capable of hybridising to the 3' adaptor molecule.

20 19. A set of oligonucleotides of known sequence with blocked 3' ends (3' adaptor molecules), wherein the 3' adaptor molecules comprise one or more degenerate nucleotides, for use in a method as defined any one of claims 2, 3, 5, 7, 8, 9 or 18.

25 20. A set of oligonucleotides of known sequence with blocked 5' ends (5' adaptor molecules), wherein the 5' adaptor molecules comprise one or more degenerate nucleotides, for use in a method as defined any one of claims 2, 3, 4, 5, 7, 8, 9 or 18.

30 21. A set of oligonucleotides of known sequence with blocked 3' ends (3' adaptor molecules) and a set of oligonucleotides of known sequence with blocked 5' ends (5' adaptor molecules), wherein the 3' and 5' adaptor molecules comprise one or

more degenerate nucleotides, for use in a method as defined any one of claims 2 to 5, 7, 8, 9 or 18.

22. A set of oligonucleotides of known sequence with blocked 3' ends (3' adaptor molecules), wherein the 3' adaptor molecules comprise one or more degenerate nucleotides, wherein said oligonucleotides are preferentially ligatable to a target sequence, for use in a method as defined in any one of claims 10 to 17.

23. A set of oligonucleotides of known sequence with blocked 5' ends (5' adaptor molecules), wherein the 5' adaptor molecules comprise one or more degenerate nucleotides, wherein said oligonucleotides are preferentially ligatable to a target sequence, for use in a method as defined in any one of claims 10 to 17.

24. A set of oligonucleotides of known sequence with blocked 3' ends (3' adaptor molecules) and a set of oligonucleotides of known sequence with blocked 5' ends (5' adaptor molecules), wherein the 3' and 5' adaptor molecules comprise one or more degenerate nucleotides, wherein said oligonucleotides are preferentially ligatable to a target sequence, for use in a method as defined in any one of claims 10 to 17.

20

CLAIMS

1. A method of reducing the sequence bias of a sequencing technique involving adaptor ligation, the method comprising:

- 5 (a) providing a set of single-stranded oligonucleotides of known sequence with blocked 3' ends (3' adaptor molecules) and a set of single-stranded oligonucleotides of known sequence with blocked 5' ends (5' adaptor molecules), wherein the 3' and 5' adaptor molecules comprise one or more degenerate nucleotides;
- 10 (b) ligating the 3' adaptor molecules to the 3' ends of the target nucleic acid molecules using a ligase;
- (c) ligating the 5' adaptor molecules to the 5' ends of the target nucleic acid molecules from step (b) using a ligase; and
- (d) determining the sequence of the target nucleic acid molecules obtained
- 15 in step (c) using a primer capable of hybridising to the complement of the 5' adaptor molecule and a primer capable of hybridising to the 3' adaptor molecule.

2. A method of detecting a target nucleic acid molecule in a library of nucleic acid

20 molecules, the method comprising:

- (a) providing a set of single-stranded oligonucleotides of known sequence with blocked 3' ends (3' adaptor molecules) and a set of single-stranded oligonucleotides of known sequence with blocked 5' ends (5' adaptor molecules), wherein the 3' and 5' adaptor molecules comprise one or more
- 25 degenerate nucleotides and are ligatable to the target nucleic acid molecule;
- (b) ligating the 3' adaptor molecule to the 3' ends of the nucleic acid molecules in the library of nucleic acid molecules using a ligase;
- (c) ligating the 5' adaptor molecule to the 5' ends of nucleic acid molecules
- 30 from step (b) using a ligase;
- (d) creating an amplified library of nucleic acid molecules by PCR of the nucleic acid molecules obtained in step (c) using a primer capable of

hybridising to the complement of the 5' adaptor molecule and a primer capable of hybridising to the 3' adaptor molecule;

(e) sequencing the resulting amplified library of nucleic acid molecules; and

5 (f) analysing the sequencing results to determine whether the target nucleic acid molecule is present in the library of nucleic acid molecules.

3. The method according to claim 2, wherein the target nucleic acid is associated with a disease or pre-disease state.

10

4. The method according to claim 2, wherein the target nucleic acid is associated with a particular organism.

5. The method according to claim 2, wherein the target nucleic acid is associated with a particular tissue type.

15

6. The method according to claim 2, wherein the target nucleic acid is associated with a particular developmental stage.

7. The method according to any one of claims 1-6, wherein the nucleic acid molecules are RNA molecules.

20

8. The method according to any one of claims 1-6, wherein the nucleic acid molecules are DNA molecules.

25

9. A method of generating a cDNA library from a library of RNA molecules, the method comprising:

(a) providing a set of single-stranded oligonucleotides of known sequence with blocked 3' ends (3' adaptor molecules) and a set of single-stranded

30

oligonucleotides of known sequence with blocked 5' ends (5' adaptor molecules), wherein the 3' and 5' adaptor molecules comprise one or more degenerate nucleotides;

- (b) ligating the 3' adaptor molecules to the 3' ends of the RNA molecules using a ligase;
- (c) ligating the 5' adaptor molecules to the 5' ends of the RNA molecules from step (b) using a ligase;
- 5 (d) creating hybrid RNA/DNA template molecules from the RNA molecules obtained in step (c) using a reverse transcriptase enzyme and a primer capable of hybridising to the 3' adaptor molecule; and
- (e) creating a cDNA library by PCR of the hybrid RNA/DNA template molecules obtained in step (d) using a primer capable of hybridising to the complement of the 5' adaptor molecule and a primer capable of hybridising to the 3' adaptor molecule.
- 10
10. A set of single-stranded oligonucleotides of known sequence with blocked 3' ends (3' adaptor molecules) and a set of single-stranded oligonucleotides of
- 15 known sequence with blocked 5' ends (5' adaptor molecules), wherein the 3' and 5' adaptor molecules comprise one or more degenerate nucleotides, for use in a method as defined in and one of claims 1-9.