

CLAIMS

WHAT IS CLAIMED IS:

1. A method for preparing a polynucleotide library, the method comprising:
 - a. in a first tailing reaction, adding a first tail to each of a plurality of target polynucleotides by template-independent polymerization, wherein the first tailing reaction comprises a first adapter comprising an overhang that hybridizes to the first tail;
 - b. in a first ligation reaction, ligating a strand of the first adapter to the first tail;
 - c. amplifying target polynucleotides comprising the strand of the first adapter by extending a first primer hybridized to the strand of the first adapter;
 - d. in a second tailing reaction, adding a second tail to each of a plurality of the amplified target polynucleotides by template-independent polymerization, wherein the second tailing reaction comprises a second adapter comprising an overhang that hybridizes to the second tail; and
 - e. in a second ligation reaction, ligating a strand of the second adapter to the second tail.
2. The method of claim 1, wherein the method comprises one or more of: (a) fragmenting polynucleotides to produce the target polynucleotides; (b) dephosphorylation of one or both ends of the target polynucleotides; and (c) denaturing double-stranded polynucleotides to single-stranded polynucleotides to produce the target polynucleotides.
3. The method of any one of the preceding claims, wherein the plurality of target polynucleotides comprises single-stranded DNA.
4. The method of any one of the preceding claims, wherein the target polynucleotides comprise cell-free polynucleotides, or amplification products thereof.
5. The method of any one of the preceding claims, wherein the target polynucleotides comprise single-stranded cell-free DNA (cfDNA).
6. The method of any one of the preceding claims, wherein the amount of target polynucleotides in the first tailing reaction is about 0.1-500 ng, 1-100 ng, or 5-50 ng.
7. The method of any one of the preceding claims, wherein the target polynucleotides have an average length of about 50 to 600 nucleotides.
8. The method of any one of the preceding claims, wherein the target polynucleotides are treated prior to step (b) to differentially modify methylated cytosines or unmethylated cytosines.

9. The method of claim 8, wherein the differentially modifying comprises treating the target polynucleotides with bisulfite.
10. The method of any one of the preceding claims, wherein the template-independent polymerization is catalyzed by a polymerase.
11. The method of claim 10, wherein the polymerase is a terminal deoxynucleotidyl transferase (TdT).
12. The method of any one of claims 1-11, wherein the first tail comprises a sequence that is different from the second tail.
13. The method of any one of claims 1-11, wherein the first tail and the second tail comprise the same sequence.
14. The method of any one of the preceding claims, wherein the first tail, the second tail, or both consist of one or two types of nucleotides.
15. The method of any one of the preceding claims, wherein the first tail, the second tail, or both are selected from the group consisting of poly-A, poly-C, and poly-C/T.
16. The method of any one of the preceding claims, wherein at least one of the tails consists of two types of nucleotides polymerized from a pool of the two types of nucleotides, wherein the two types of nucleotides in the pool are present in same or different amounts.
17. The method of claim 16, wherein the two types of nucleotides in the pool are in a ratio of about 9:1, 5:1, 3:1, or 1:1.
18. The method of any one of the preceding claims, wherein the first adapter and the second adapter comprise double-stranded regions that are different in polynucleotide sequence.
19. The method of any one of the preceding claims, wherein the amplifying comprises linear amplification.
20. The method of any one of the preceding claims, wherein the overhang of the first and/or second adapter is a 3'-overhang.
21. The method of any one of the preceding claims, wherein the overhang of the first and/or second adapter is 6 to 12 nucleotides in length.
22. The method of any one of the preceding claims, wherein (i) the first tailing reaction and the first ligation reaction occur in the same reaction mixture, and/or (ii) the second tailing reaction and the second ligation reaction occur in the same reaction mixture.
23. The method of any one of the preceding claims, further comprising amplifying target polynucleotides comprising the strand of the second adapter by extending a second primer hybridized to the strand of the second adapter.

24. The method of claim 23, wherein the sequence of the first primer that hybridizes with the strand of the first adapter is different from the sequence of the second primer that hybridizes with the second adapter.
25. The method of claim 23 or 24, wherein amplification with the primer hybridized to the strand of the second adapter is an exponential amplification.
26. The method of any one of claims 23-25, further comprising an amplification reaction with a third primer and a fourth primer, wherein (i) the third primer hybridizes to a complement of at least a portion of the first primer, and (ii) the fourth primer hybridizes to a complement of at least a portion of the second primer.
27. The method of claim 26, wherein the hybridizable sequence of the third primer is different from the hybridizable sequence of the first primer, and/or the hybridizable sequence of the fourth primer is different from the hybridizable sequence of the second primer.
28. The method of claim 26 or 27, wherein the sequences of the third primer and the fourth primer are different.
29. The method of any one of claims 26-28, wherein the third primer, the fourth primer, or both comprise an index sequence that identifies a sample source of the target polynucleotides.
30. The method of any one claims 23-25, further comprising sequencing amplification products of the amplification comprising the second primer.
31. The method of any one of claims 26-29, further comprising sequencing amplification products of the amplification comprising the third and fourth primer.
32. The method of claim 31, further comprising grouping sequencing reads according to the index sequence.
33. The method of claim 31 or 32, wherein sequencing comprises detecting a sequence variant or a difference in nucleotide methylation, relative to a reference sequence.
34. A composition for use in the method of any one of the preceding claims.
35. A polynucleotide produced according to the method of any one of the preceding claims.
36. A kit for preparing a polynucleotide library, the kit comprising:
 - a. a template-independent polymerase;
 - b. a first pool of nucleotides that can be polymerized by the template-independent polymerase;
 - c. a second pool of nucleotides that can be polymerized by the template-independent polymerase;

- d. a first adapter comprising an overhang that is hybridizable to tails formed by polymerizing the first pool of polynucleotides; and
 - e. a second adapter comprising an overhang that is hybridizable to tails formed by polymerizing the second pool of polynucleotides, wherein the second adapter comprises a different sequence than the first adapter.
37. The kit of claim 36, wherein the template-independent polymerase is a terminal deoxynucleotidyl transferase (TdT).
38. The kit of claim 36 or 37, wherein at least one of the first pool and the second pool contains at least one type of nucleotide not present in the other pool.
39. The kit of claim 36 or 37, wherein the first pool and the second pool comprise the same one or more types of nucleotides.
40. The kit of any one of claims 36-38, wherein the first pool, the second pool, or both consist of one or two types of nucleotides.
41. The kit of any one of claims 36-40, wherein the first pool, the second pool, or both are selected from the group consisting of (i) a pool of dATP, (ii) a pool of dCTP, and (iii) a pool of dCTP and dTTP.
42. The kit of any one of claims 36-41, wherein at least one of the first pool and the second pool consists of two types of nucleotides that are present in same or different amounts.
43. The kit of claim 42, wherein the two types of nucleotides in the pool are in a ratio of about 9:1, 5:1, 3:1, or 1:1.
44. The kit of any one of claims 36-43, wherein the first adapter and the second adapter comprise double-stranded regions that are different in polynucleotide sequence.
45. The kit of any one of claims 36-44, wherein the overhang of the first and/or second adapter is a 3'-overhang.
46. The kit of any one of claims 36-45, wherein the overhang of the first and/or second adapter is 6 to 12 nucleotides in length.
47. The kit of any one of claims 36-46, further comprising a first primer that is hybridizable to a strand of the first adapter under conditions for a primer extension reaction.
48. The kit of any one of claims 36-47, further comprising a second primer that is hybridizable to a strand of the second adapter under conditions for a primer extension reaction.
49. The kit of claim 48, wherein the sequence of the first primer that is hybridizable to the strand of the first adapter is different from the sequence of the second primer that is hybridizable to the second adapter.

50. The kit of claim 48 or 49, further comprising a third primer and a fourth primer, wherein (i) the third primer is hybridizable to a complement of at least a portion of the first primer under conditions for a primer extension reaction, and (ii) the fourth primer is hybridizable to a complement of at least a portion of the second primer under conditions for a primer extension reaction.
51. The kit of claim 50, wherein the hybridizable sequence of the third primer is different from the hybridizable sequence of the first primer, and/or the hybridizable sequence of the fourth primer is different from the hybridizable sequence of the second primer.
52. The kit of claim 50 or 51, wherein the hybridizable sequence of the third primer hybridizes 5' with respect to the hybridizable sequence of the first primer, and/or the hybridizable sequence of the fourth primer hybridizes 5' with respect to the hybridizable sequence of the second primer.
53. The kit of any one of claims 50-52, wherein the sequences of the third primer and fourth primer are different.
54. The kit of any one of claims 50-53, wherein the third primer, the fourth primer, or both comprise an index sequence that identifies a sample source of the target polynucleotides.
55. A method for preparing a polynucleotide library, the method comprising:
- a. in a first tailing reaction, adding a first tail to each of a plurality of target polynucleotides by template-independent polymerization, wherein the first tailing reaction comprises a first adapter comprising an overhang that hybridizes to the first tail;
 - b. in a first ligation reaction, ligating a strand of the first adapter to the first tail;
 - c. amplifying target polynucleotides comprising the strand of the first adapter by extending a first primer hybridized to the strand of the first adapter; and
 - d. in a second ligation reaction, ligating a strand of a second adapter to the amplified target polynucleotides.
56. The method of claim 55, wherein the second ligation reaction comprises, in a second tailing reaction, adding a second tail to each of a plurality of the amplified target polynucleotides by template-independent polymerization.
57. The method of claim 56, wherein the second tailing reaction comprises a second adapter comprising an overhang that hybridizes to the second tail.
58. The method of claim 57, wherein, in the second ligation reaction, ligating a strand of the second adapter to the second tail.
59. The method of claim 55, wherein the second ligation reaction comprises a second adapter comprising an overhang that hybridizes to the amplified target polynucleotides.

60. The method of any one of claims 55-59, wherein the method comprises one or more of:
(a) fragmenting polynucleotides to produce the target polynucleotides; (b)
dephosphorylation of one or both ends of the target polynucleotides; and (c) denaturing
double-stranded polynucleotides to single-stranded polynucleotides to produce the target
polynucleotides.
61. The method of any one of claims 55-60, wherein the plurality of target polynucleotides
comprises single-stranded DNA.
62. The method of any one of claims 55-61, wherein the target polynucleotides comprise
cell-free polynucleotides, or amplification products thereof.
63. The method of any one of claims 55-62, wherein the target polynucleotides comprise
single-stranded cell-free DNA (cfDNA).
64. The method of any one claims 55-63, wherein the amount of target polynucleotides in
the first tailing reaction is about 0.1-500 ng, 1-100 ng, or 5-50 ng.
65. The method of any one of claims 55-69, wherein the target polynucleotides have an
average length of about 50 to 600 nucleotides.
66. The method of any one of claims 55-65, wherein the target polynucleotides are treated
prior to step (b) to differentially modify methylated cytosines or unmethylated cytosines.
67. The method of claim 66, wherein the differentially modifying comprises treating the
target polynucleotides with bisulfite.
68. The method of any one of claims 55-67, wherein the template-independent
polymerization is catalyzed by a polymerase.
69. The method of claim 68, wherein the polymerase is a terminal deoxynucleotidyl
transferase (TdT).
70. The method of any one of claims 55-69, wherein the first tail comprises a sequence that
is different from the second tail.
71. The method of any one of claims 55-69, wherein the first tail and the second tail
comprise the same sequence.
72. The method of any one of claims 55-71, wherein the first tail, the second tail, or both
consist of one or two types of nucleotides.
73. The method of any one of claims 55-72, wherein the first tail, the second tail, or both are
selected from the group consisting of poly-A, poly-C, and poly-C/T.
74. The method of any one of claims 55-73, wherein at least one of the tails consists of two
types of nucleotides polymerized from a pool of the two types of nucleotides, wherein
the two types of nucleotides in the pool are present in same or different amounts.

75. The method of claim 74, wherein the two types of nucleotides in the pool are in a ratio of about 9:1, 7:1, 5:1, 3:1, or 1:1.
76. The method of any one of claims 55-75 wherein the second tailing reaction is omitted.
77. The method of any one of claims 55-76, wherein the first adapter and the second adapter comprise double-stranded regions that are different in polynucleotide sequence.
78. The method of any one of claims 55-77, wherein the amplifying comprises linear amplification.
79. The method of any one of claims 55-78, wherein the overhang of the first and/or second adapter is a 3'-overhang.
80. The method of any one of claims 55-79, wherein the first and/or second adapter have both a 3'-overhang and a 5'-overhang.
81. The method of any one of claims 55-80, wherein the 3'-overhang of the first and/or second adapter is 6 to 12 nucleotides in length.
82. The method of any one claims 55-81, wherein the 5'-overhang of the first and/or second adapter is 2 to 6 nucleotides in length.
83. The method of any one claims 55-82, wherein (i) the first tailing reaction and the first ligation reaction occur in the same reaction mixture, and/or (ii) the second tailing reaction and the second ligation reaction occur in the same reaction mixture.
84. The method of any one claims 55-83, further comprising amplifying target polynucleotides comprising the strand of the second adapter by extending a second primer hybridized to the strand of the second adapter.
85. The method of claim 84, wherein the sequence of the first primer that hybridizes with the strand of the first adapter is different from the sequence of the second primer that hybridizes with the second adapter.
86. The method of claim 84 or 85, wherein amplification with the primer hybridized to the strand of the second adapter is an exponential amplification.
87. The method of any one of claims 84-86, further comprising an amplification reaction with a third primer and a fourth primer, wherein (i) the third primer hybridizes to a complement of at least a portion of the first primer, and (ii) the fourth primer hybridizes to a complement of at least a portion of the second primer.
88. The method of claim 87, wherein the hybridizable sequence of the third primer is different from the hybridizable sequence of the first primer, and/or the hybridizable sequence of the fourth primer is different from the hybridizable sequence of the second primer.

89. The method of claim 87 or 88, wherein the sequences of the third primer and the fourth primer are different.
90. The method of any one of claims 87-89, wherein the third primer, the fourth primer, or both comprise an index sequence that identifies a sample source of the target polynucleotides.
91. The method of any one claims 84-86, further comprising sequencing amplification products of the amplification comprising the second primer.
92. The method of any one of claims 87-90, further comprising sequencing amplification products of the amplification comprising the third and fourth primer.
93. The method of claim 92, further comprising grouping sequencing reads according to the index sequence.
94. A composition for use in the method of any one of claims 55-93.
95. A polynucleotide produced according to the method of any one of claims 55-93.
96. A kit for preparing a polynucleotide library, the kit comprising:
 - a. a template-independent polymerase;
 - b. a first pool of nucleotides that can be polymerized by the template-independent polymerase;
 - c. a second pool of nucleotides that can be polymerized by the template-independent polymerase;
 - d. a first adapter comprising an overhang that is hybridizable to tails formed by polymerizing the first pool of polynucleotides; and
 - e. a second adapter comprising an overhang that is hybridizable to the amplified target polynucleotides.
97. The kit of claim 96, wherein the template-independent polymerase is a terminal deoxynucleotidyl transferase (TdT).
98. The kit of claim 96 or 97, wherein at least one of the first pool and the second pool contains at least one type of nucleotide not present in the other pool.
99. The kit of claim 96 or 97, wherein the first pool and the second pool comprise the same one or more types of nucleotides.
100. The kit of any one of claims 96-98, wherein the first pool, the second pool, or both consist of one or two types of nucleotides.
101. The kit of any one of claims 96-100, wherein the first pool, the second pool, or both are selected from the group consisting of (i) a pool of dATP, (ii) a pool of dCTP, and (iii) a pool of dCTP and dTTP.

102. The kit of any one of claims 96-101, wherein at least one of the first pool and the second pool consists of two types of nucleotides that are present in same or different amounts.
103. The kit of claim 102, wherein the two types of nucleotides in the pool are in a ratio of about 9:1, 7:1, 5:1, 3:1, or 1:1.
104. The kit of any one of claims 96-103, wherein the first adapter and the second adapter comprise double-stranded regions that are different in polynucleotide sequence.
105. The kit of any one of claims 96-104, wherein the overhang of the first and/or second adapter is a 3'-overhang.
106. The kit of any one of claims 96-104, wherein the first and/or second adapter have both a 3'-overhang and a 5'-overhang.
107. The kit of any one of claims 96-105, wherein the 3'-overhang of the first and/or second adapter is 6 to 12 nucleotides in length.
108. The kit of any one of claims 96-106, wherein the 5'-overhang of the first and/or second adapter is 2 to 6 nucleotides in length.
109. The kit of any one of claims 96-107, further comprising a first primer that is hybridizable to a strand of the first adapter under conditions for a primer extension reaction.
110. The kit of any one of claims 96-109, further comprising a second primer that is hybridizable to a strand of the second adapter under conditions for a primer extension reaction.
111. The kit of claim 110, wherein the sequence of the first primer that is hybridizable to the strand of the first adapter is different from the sequence of the second primer that is hybridizable to the second adapter.
112. The kit of claim 110 or 111, further comprising a third primer and a fourth primer, wherein (i) the third primer is hybridizable to a complement of at least a portion of the first primer under conditions for a primer extension reaction, and (ii) the fourth primer is hybridizable to a complement of at least a portion of the second primer under conditions for a primer extension reaction.
113. The kit of claim 112, wherein the hybridizable sequence of the third primer is different from the hybridizable sequence of the first primer, and/or the hybridizable sequence of the fourth primer is different from the hybridizable sequence of the second primer.
114. The kit of claim 112 or 113, wherein the hybridizable sequence of the third primer hybridizes 5' with respect to the hybridizable sequence of the first primer, and/or the hybridizable sequence of the fourth primer hybridizes 5' with respect to the hybridizable sequence of the second primer.

115. The kit of any one of claims 112-114, wherein the sequences of the third primer and fourth primer are different.
116. The kit of any one of claims 112-115, wherein the third primer, the fourth primer, or both comprise an index sequence that identifies a sample source of the target polynucleotides.