CHARACTERIZING MARKER-SPECIFIC IDENTIFIERS FROM HIGHLY MULTIPLEXED FLAP CLEAVAGE ASSAYS

PRIORITY STATEMENT

This application claims priority to U.S. Provisional Application No. 63/581,624, filed September 8, 2023, and to U.S. Provisional Application No. 63/593,807, filed October 27, 2023, the entire contents of each of which are incorporated herein by reference for all purposes.

FIELD OF THE INVENTION

Provided herein is technology relating to compositions and methods for detecting multiple target sequences using flap oligonucleotides and ligatable molecules, including circularizable vector molecules (*e.g.*, padlock probe or dumbbell probes) and pairs of oligonucleotides (*e.g.*, "vector pairs") that form a linear construct upon ligation to a flap oligonucleotide. In some embodiments, a bridge oligonucleotide, *e.g.*, a DNA, RNA, or chimerical oligonucleotide, is used to align the ends of the vector and cleaved flap sequences to form one or more ligatable nicks. The methods described herein find use in methods of detecting mixtures of cleaved probe flaps, *e.g.*, cleaved flaps produced in multiplexed and highly-multiplexed flap cleavage assays, including multiplex PCR-flap assays, using circular and/or circularizable molecules, or using separate oligonucleotides or vector pairs to form linear molecules upon ligation. Embodiments of the technology comprise amplification and/or sequencing techniques for detecting and/or counting cleaved flaps produced in flap cleavage assays.

BACKGROUND OF THE INVENTION

Detection of target nucleic acids, *e.g.,* for diagnostic purposes, typically requires a high degree of specificity, *e.g.,* to distinguish between nucleic acids that may differ by a single nucleotide, and a high degree of sensitivity, *e.g.,* to detect target nucleic acids that may be present in very low copy numbers. There is a need in the field for assays that combine specific

recognition of target nucleic acids with signal amplification to produce sensitive reporting of the presence of the target nucleic acids.

SUMMARY OF THE INVENTION

The technology herein provides methods, compositions, kits, systems, reagents, and reaction mixtures, for characterization of samples, *e.g.,* nucleic acid samples from subjects, in particular by characterizing cleaved flaps that comprise marker-specific identifiers, *e.g.*, cleaved flaps produced in multiplexed flap cleavage assays.

Disclosed herein is a method comprising:

a) providing a reaction mixture comprising or suspected of comprising at least one target nucleic acid, at least one flap oligonucleotide, and a flap endonuclease, wherein each flap oligonucleotide comprises:

i) a target-specific portion that anneals to a target nucleic acid, if the target nucleic acid is present in the reaction mixture, and

ii) a 5′ flap portion, comprising a sequence of nucleotides indicative of the target nucleic acid to which the target-specific portion anneals;

wherein, for target nucleic acids present in the reaction mixture, invasive cleavage structures comprising a target nucleic acid annealed to a flap oligonucleotide are formed;

wherein, in the reaction mixture, invasive cleavage structures are cleaved by the flap endonuclease to produce at least one cleaved flap;

b) providing at least one ligated molecule, wherein the ligated molecule comprises sequence of the cleaved flap, and/or a sequence complementary to the cleaved flap, and

c) characterizing the at least one ligated molecule to characterize the at least one target nucleic acid present in the reaction mixture.

Disclosed herein is a method comprising:

a) providing a reaction mixture comprising or suspected of comprising a population of different target nucleic acids, a plurality of flap oligonucleotides, and a flap endonuclease, wherein each flap oligonucleotide comprises:

i) a target-specific portion that anneals to a target nucleic acid, if the target nucleic acid is present in the reaction mixture, and

ii) a 5′ flap portion, comprising a sequence of nucleotides indicative of the target nucleic acid to which the target-specific portion anneals;

wherein, for target nucleic acids present in the reaction mixture, invasive cleavage structures comprising a target nucleic acid annealed to a flap oligonucleotide are formed;

wherein, in the reaction mixture, the invasive cleavage structures are cleaved by the flap endonuclease to produce population of cleaved flaps;

b) providing a population of ligated molecules, wherein the ligated molecules comprise sequences of the population of cleaved flaps, and/or sequences complementary to the population of cleaved flaps, and

c) characterizing the population of ligated molecules to characterize the population of different target nucleic acids present in the reaction mixture.

Disclosed herein is a composition comprising:

i) one or both of:

a) at least one cleaved flap; and

b) at least one ligated molecule comprising a cleaved flap sequence or a complement thereof, wherein the cleaved flap sequence or complement thereof comprises a sequence of nucleotides indicative of a target nucleic acid;

and

ii) a ligase enzyme.

Disclosed herein is a composition comprising:

i) one or both of:

a) a population of cleaved flaps; and

b) a population of ligated molecules, each ligated molecule comprising a cleaved flap sequence or a complement thereof, wherein each cleaved flap sequence or complement thereof comprises a sequence of nucleotides indicative of a target nucleic acid;

and

ii) a ligase enzyme.

Disclosed herein is a method, comprising:

a) providing in a mixture a bridge oligonucleotide, a flap sequence oligonucleotide, preferably a cleaved flap oligonucleotide, a first ligatable vector molecule and second ligatable vector molecule comprising a 5′ blocking group, wherein the bridge oligonucleotide comprises:

i) an inner segment that is complementary to the flap sequence oligonucleotide,

ii) a 3′ end that is complementary to a 3′ end of the first ligatable vector molecule, and

iii) a 5′ end that is complementary to a 5′ end of the second ligatable vector molecule,

wherein, in the mixture, the bridge oligonucleotide is hybridized to the flap sequence oligonucleotide and to the first and second ligatable molecules to form a complex comprising:

I) a first ligatable nick comprising the 3′ end of the first ligatable molecule and a 5′ end of the flap sequence oligonucleotide, and

II) an invasive cleavage substrate comprising a 3′ end of the flap sequence oligonucleotide and the 5′ end of the second ligatable vector molecule comprising the 5′ blocking group;

b) treating the complex with a flap endonuclease in conditions wherein cleavage of the invasive cleavage substrate cleaves off the 5′ blocking group of the second ligatable vector molecule and produces a second ligatable nick; and

c) treating the complex with a ligase under conditions wherein the first ligatable nick and the second ligatable nick are ligated to form a ligated molecule.

Exemplary embodiments of the technology include but are not limited to the following:

1. A method comprising:

a) providing a reaction mixture comprising or suspected of comprising a population of different target nucleic acids, a plurality of flap oligonucleotides, and a flap endonuclease, wherein each flap oligonucleotide comprises:

i) a target-specific portion that anneals to a target nucleic acid, if the target nucleic acid is present in the reaction mixture, and

ii) a 5′ flap portion, comprising a sequence of nucleotides indicative of the target nucleic acid to which the target-specific portion anneals;

wherein, for target nucleic acids present in the reaction mixture, invasive cleavage structures comprising a target nucleic acid annealed to a flap oligonucleotide are formed;

wherein, in the reaction mixture, the invasive cleavage structures are cleaved by the flap endonuclease to produce population of cleaved flaps;

b) providing a population of ligated molecules, wherein the ligated molecules comprise sequences of the population of cleaved flaps, and/or sequences complementary to the population of cleaved flaps, and

c) characterizing the population of ligated molecules to characterize the population of different target nucleic acids present in the reaction mixture.

2. The method of embodiment 1, wherein the ligated molecules are circular molecules.

3. The method of embodiment 1, wherein the ligated molecules are linear molecules.

4. The method of embodiment 1, wherein providing the population of ligated molecules comprises treating the population of cleaved flaps with a preparation of ligatable vector molecules under conditions wherein cleaved flaps are ligated to the ligatable vector molecules to form the population of ligated molecules, each ligated molecule comprising a vector molecule sequence and a cleaved flap sequence.

5. The method of any one of embodiments 1-4, wherein the reaction mixture is an amplification reaction mixture, and wherein the population of different target nucleic acids comprises amplified nucleic acids produced in the amplification reaction mixture.

6. The method of embodiment 5, wherein the amplification reaction mixture is a PCR-flap assay reaction mixture.

7. The method of any one of embodiments 1-6, wherein characterizing the population of ligated molecules comprises counting different cleaved flap sequences, or complements thereof, present in the population of ligated molecules.

8. The method of embodiment 7, wherein characterizing the population of ligated molecules comprises sequencing cleaved flap sequences, or the complements thereof, in the population of ligated molecules.

9. The method of any one of embodiments 1-8, wherein the flap endonuclease is a FEN-1 endonuclease.

10. The method of embodiment 9, wherein the FEN-1 endonuclease is a strict FEN-1 endonuclease, and wherein the reaction mixture comprises a Mg^{++} flap assay buffer.

11. The method of embodiment 10, wherein the strict FEN-1 endonuclease is selected from the group consisting of *Archaeoglobus fulgidus* (Afu) FEN-1, *Pyrococcus furiosus* (Pfu) FEN-1, and *Archaeoglobus veneficus* (Ave) FEN-1, and variants thereof, and CLEAVASE 2.0 nuclease.

12. The method of any one of embodiments 1-11, wherein each ligated molecule in the population of ligated molecules comprises one or more of:

i) a first bridge oligonucleotide-complementary arm at a 3′ end of a first vector sequence and a second bridge oligonucleotide-complementary arm at a 5′ end of a second vector sequence, wherein the first bridge oligonucleotide-complementary arm has a different nucleotide sequence than the second bridge oligonucleotide-complementary arm;

ii) a pair of amplification primer binding sites configured to amplify a portion of a ligated molecule that comprises a cleaved flap sequence;

iii) a primer binding site configured to amplify a circular ligated molecule by rolling circle amplification;

iv) at least one unique molecular tag;

v) a polynucleotide linker; and/or

vi) a polymerization-terminating molecule, preferably an internal spacer.

13. The method of any one of embodiments 1-12, further comprising a step of amplifying regions of the ligated molecules comprising cleaved flap sequences to form a population of flap amplicons.

14. The method of embodiment 13, further comprising sequencing flap amplicons to produce sequencing reads.

15. The method of any one of embodiments 4-14, wherein the ligatable vector molecules comprise one or more of dumbbell probes, padlock probes, vector pairs, and/or linear vector molecules.

16. The method of any one of embodiments 4-15, wherein the ligatable vector molecules comprise vector pairs, wherein each vector pair comprises a first bridge oligonucleotide-complementary arm at a 3′ end of a first vector sequence and a second bridge oligonucleotide-complementary arm at a 5′ end of a second vector sequence, and wherein step b) comprises contacting the population of cleaved flaps with the preparation of ligatable vector molecules and a population of bridge oligonucleotides, wherein each bridge oligonucleotide comprises an inner segment that is complementary to a cleaved flap sequence, a 3′ end that is complementary to the 3′ end of the first vector sequence, and a 5′ end that is complementary to the 5′ end of the second vector sequence.

17. The method of embodiment 16, wherein ligatable vector molecules comprising the 5′ end of the second vector sequence each comprise a 5′ blocking group.

18. The method of any one of embodiments 4-15, wherein the ligatable vector molecules are dumbbell probes, wherein each dumbbell probe comprises a bridge sequence that is complementary to a cleaved flap sequence.

19. The method of any one of embodiments 1-15 and 17-18, wherein the ligated molecules comprise circular molecules, the method further comprising a step of replicating the circular molecules comprising cleaved flap sequences using rolling circle replication, to form a population of flap replicons.

20. The method of any one of embodiments 1-19, wherein the plurality of flap oligonucleotides in a reaction mixture comprises at least 10 unique flap oligonucleotides.

21. The method of embodiment 20, wherein the plurality of flap oligonucleotides in a reaction mixture comprises at least 20 unique flap oligonucleotides.

22. The method of embodiment 20, wherein the plurality of flap oligonucleotides in a reaction mixture comprises at least 30 unique flap oligonucleotides.

23. The method of embodiment 20, wherein the plurality of flap oligonucleotides in a reaction mixture comprises 40 to 1000 unique flap oligonucleotides.

24. The method of any one of embodiments 1-23, wherein the target-specific portions of flap oligonucleotides in the plurality of flap oligonucleotides in the reaction mixture are at least 6 nucleotides in length, preferably at least 7, preferably at least 8, preferably at least 9, preferably at least 10, preferably at least 11, preferably at least 12 nucleotides in length.

25. The method of embodiment 24, wherein the target-specific portions of the flap oligonucleotides in the plurality of flap oligonucleotides in the reaction mixture are 13 to 100 nucleotides in length.

26. The method of any one of embodiments 1-25, wherein the target nucleic acid comprises one or more of cDNA, amplified DNA, genomic DNA, and circulating cell-free DNA.

27. The method of any one of embodiments 1-26, wherein the target nucleic acid comprises DNA treated with a methylation-specific reagent.

28. The method of embodiment 27, wherein the reaction mixture further comprises oligonucleotides specific for a reference nucleic acid.

29. The method of embodiment 28, wherein the oligonucleotides comprise a flap oligonucleotide, an invasive oligonucleotide, and optionally a non-invasive oligonucleotide.

30. The method of embodiment 28 or embodiment 29, wherein a concentration of one or more of the oligonucleotides specific for the reference nucleic acid is altered in the reaction mixture relative to a concentration of corresponding oligonucleotides specific for the target nucleic acids in the reaction mixture, and/or wherein one or more of the oligonucleotides specific for the reference nucleic acid binds to the reference nucleic acid with one or more mismatched base pairings.

31. The method of embodiment 30, wherein the concentration of the one or more of the oligonucleotides specific for the reference nucleic acid is reduced in the reaction mixture relative to the concentration of the corresponding oligonucleotides specific for the target nucleic acids in the reaction mixture.

32. The method of any one of embodiments 28 to 31, wherein the reference nucleic acid comprises methylated *B3GALT6* DNA.

33. A composition comprising:

i) one or both of:

a) a population of cleaved flaps; and

b) a population of ligated molecules, each ligated molecule comprising a cleaved flap sequence or a complement thereof, wherein each cleaved flap sequence or complement thereof comprises a sequence of nucleotides indicative of a target nucleic acid;

and

ii) a ligase enzyme.

34. The composition of embodiment 33, further comprising a population of bridge oligonucleotides, wherein each bridge oligonucleotide comprises a 5′ end sequence that is complementary to a 5′ end of a cleaved flap sequence, and a 3′ end sequence that is complementary a 3′ end of the cleaved flap sequence, wherein hybridization of the bridge oligonucleotide to a cleaved flap, if present in the composition, forms a ligatable nick.

35. The composition of embodiment 33, further comprising ligatable vector molecules, wherein each ligated molecule comprises at least one ligatable vector molecule sequence.

36. The composition of embodiment 33 or embodiment 35, further comprising a population of bridge oligonucleotides, wherein each bridge oligonucleotide comprises an inner segment that is complementary to a cleaved flap sequence, a 3′ end that is complementary to a 3′ end of a first vector sequence, and a 5′ end that is complementary to the 5′ end of a second vector sequence.

37. The composition of embodiment 36, further comprising one or more of the following:

i) a first ligatable vector molecule and second ligatable vector molecule, the second ligatable vector molecule comprising a 5′ blocking group, wherein in the composition, the bridge oligonucleotide is hybridized to the cleaved flap and to the first and second ligatable vector molecules in a complex comprising:

I) a first ligatable nick comprising the 3′ end of the first ligatable molecule and a 5′ end of the cleaved flap, and

II) an invasive cleavage substrate comprising a 3′ end of the cleaved flap and the 5′ end of the second ligatable vector molecule comprising the 5′ blocking group,

wherein the 5′ blocking group preferably comprises a moiety selected from a hexane, a non-standard nucleotide, an abasic nucleotide, a fluorophore, a flap nucleic acid sequence, a hairpin flap nucleic acid sequence, and a hydroxyl group;

ii) a bridge oligonucleotide comprising one or more of an RNA portion and a DNA portion;

iii) a bridge oligonucleotide comprising an RNA portion comprising one or more nucleotide modifications, preferably selected from 2′-O-methyl and 2′-O-methoxyethyl nucleotide modifications;

iv) a flap endonuclease, preferably a FEN-1 endonuclease, preferably a strict FEN-1 endonuclease, preferably a strict FEN-1 endonuclease selected from the group consisting of *Archaeoglobus fulgidus* (Afu) FEN-1, *Pyrococcus furiosus* (Pfu) FEN-1, and *Archaeoglobus veneficus* (Ave) FEN-1, and variants thereof, and CLEAVASE 2.0 nuclease;

v) a Mg^{++} flap assay buffer;

vi) amplification reagents;

vii) flap assay reagents; and/or

viii) a ligase comprises *Chlorella* virus PBCV-1 DNA ligase.

38. The composition of any one of embodiments 33-37, wherein each ligated molecule in the population of ligated molecules comprises one or more of:

i) a pair of amplification primer binding sites configured to amplify a portion of a ligated molecule that comprises a cleaved flap sequence;

ii) a primer binding site configured to amplify a circular ligated molecule by rolling circle amplification;

iii) at least one unique molecular tag;

iv) a polynucleotide linker; and/or

v) a polymerization-terminating molecule, preferably an internal spacer.

39. The composition of any one of embodiments 33 to 38, further comprising a population of amplified products, each amplified product comprising a target nucleic acid sequence that is associated with a cleaved flap sequence in a ligated molecule in the population of ligated molecules.

40. A method, comprising:

a) providing in a mixture a bridge oligonucleotide, a flap sequence oligonucleotide, preferably a cleaved flap oligonucleotide, a first ligatable vector molecule and second ligatable vector molecule comprising a 5′ blocking group, wherein the bridge oligonucleotide comprises:

i) an inner segment that is complementary to the flap sequence oligonucleotide,

ii) a 3′ end that is complementary to a 3′ end of the first ligatable vector molecule, and

iii) a 5′ end that is complementary to a 5′ end of the second ligatable vector molecule,

wherein in the mixture, the bridge oligonucleotide is hybridized to the flap sequence oligonucleotide and to the first and second ligatable molecules to form a complex comprising:

I) a first ligatable nick comprising the 3′ end of the first ligatable molecule and a 5′ end of the flap sequence oligonucleotide, and

II) an invasive cleavage substrate comprising a 3′ end of the flap sequence oligonucleotide and the 5′ end of the second ligatable vector molecule comprising the 5′ blocking group;

b) treating the complex with a flap endonuclease in conditions wherein cleavage of the invasive cleavage substrate cleaves off the 5′ blocking group of the second ligatable vector molecule and produces a second ligatable nick; and

c) treating the complex with a ligase under conditions wherein the first ligatable nick and the second ligatable nick are ligated to form a ligated molecule.

41. The method of embodiment 40, wherein the 5′ blocking group comprises a moiety selected from a hexane, a non-standard nucleotide, an abasic nucleotide, a fluorophore, a flap nucleic acid sequence, a hairpin flap nucleic acid sequence, and a hydroxyl group.

42. The method of embodiment 40 or 41, wherein the bridge oligonucleotide comprises one or more of an RNA portion and a DNA portion.

43. The method of embodiment 42, wherein the RNA portion comprises one or more nucleotide modifications.

44. The method of embodiment 43, wherein the one or more nucleotide modifications comprise 2′-O-methyl or 2′-O-methoxyethyl modifications.

45. The method of embodiment 43 or embodiment 44, wherein the 5′ end and an adjacent part of the inner segment of the bridge oligonucleotide comprise RNA, 2′-O-methyl RNA, or 2′-O-methoxyethyl RNA.

46. The method of embodiment 43, wherein the 3′ end and an adjacent part of the inner segment of the bridge oligonucleotide comprise DNA.

47. The method of any one of embodiments 40-46, further comprising detecting the ligated molecule in a nucleic acid detection assay.

48. The method of any one of embodiments 40-47, wherein the ligase comprises *Chlorella* virus PBCV-1 DNA ligase.

49. A method comprising:

a) providing a reaction mixture comprising or suspected of comprising a population of different target nucleic acids, a plurality of flap oligonucleotides, and a flap endonuclease, wherein each flap oligonucleotide comprises:

i) a target-specific portion that anneals to a target nucleic acid, if the target nucleic acid is present in the reaction mixture, and

ii) a 5′ flap portion, comprising a sequence of nucleotides indicative of the target nucleic acid to which the target-specific portion anneals;

wherein, for target nucleic acids present in the reaction mixture, invasive cleavage structures comprising a target nucleic acid annealed to a flap oligonucleotide are formed;

wherein, in the reaction mixture, the invasive cleavage structures are cleaved by the flap endonuclease to produce population of cleaved flaps;

b) providing a population of circular molecules, wherein the circular molecules comprise sequences of the population of cleaved flaps, and/or sequences complementary to the population of cleaved flaps, and

c) characterizing the population of circular molecules to characterize the population of different target nucleic acids present in the reaction mixture.

50. The method of embodiment 49, wherein characterizing the population of circular molecules comprises detecting hybridization of cleaved flaps to the circular molecules.

51. The method of embodiment 49, wherein providing the population of circular molecules comprises treating the population of cleaved flaps under conditions wherein 5′ and 3′ ends of individual cleaved flaps are ligated together to form circular molecules.

52. The method of embodiment 49, wherein providing the population of circular molecules comprises treating the population of cleaved flaps with a preparation of circularizable molecules under conditions wherein cleaved flaps are ligated to the circularizable molecules to form the population of circular molecules, each circular molecule comprising a circularizable molecule sequence and a cleaved flap sequence.

53. The method of any one of embodiments 49-52, wherein the reaction mixture is an amplification reaction mixture, and wherein the population of different target nucleic acids comprises amplified nucleic acids produced in the amplification reaction mixture.

54. The method of embodiment 53, wherein the amplification reaction mixture is a PCR-flap assay reaction mixture.

55. The method of any one of embodiments 49-54, wherein characterizing the population of circular molecules comprises counting different cleaved flap sequences, or complements thereof, present in the population of circular molecules.

56. The method of embodiment 55, wherein characterizing the population of circular molecules comprises sequencing cleaved flap sequences, or the complements thereof, in the population of circular molecules.

57. The method of any one of embodiments 49-56, wherein the flap endonuclease is a FEN-1 endonuclease.

58. The method of embodiment 57, wherein the FEN-1 endonuclease is a strict FEN-1 endonuclease, and wherein the reaction mixture comprises a Mg^{++} flap assay buffer.

59. The method of embodiment 58, wherein the strict FEN-1 endonuclease is selected from the group consisting of *Archaeoglobus fulgidus* (Afu) FEN-1, *Pyrococcus furiosus* (Pfu) FEN-1, and *Archaeoglobus veneficus* (Ave) FEN-1, and variants thereof, and CLEAVASE 2.0 nuclease.

60. The method of any one of embodiments 49-59, wherein each circular molecule in the population of circular molecules comprises one or more of:

i) a pair of bridge oligonucleotide-complementary arms at 5′ and 3′ termini of a circularizable molecule, wherein a first bridge oligonucleotide complementary arm has a different nucleotide sequence than a second bridge oligonucleotide complementary arm;

ii) a pair of amplification primer binding sites configured to amplify a portion of a circular molecule that comprises a cleaved flap sequence;

iii) a primer binding site configured to amplify the circular molecule by rolling circle amplification;

iv) at least one unique molecular tag;

v) a polynucleotide linker; and/or

vi) a polymerization-terminating molecule, preferably an internal spacer.

61. The method of any one of embodiments 49-60, further comprising a step of amplifying regions of the circular molecules comprising cleaved flap sequences to form a population of flap amplicons.

62. The method of embodiment 61, further comprising sequencing flap amplicons to produce sequencing reads.

63. The method of any one of embodiments 52-62, wherein the circularizable molecules comprise dumbbell probes and/or padlock probes.

64. The method of any one of embodiments 52-63, wherein the circularizable molecules each comprise a pair of bridge oligonucleotide-complementary arms at 5′ and 3′ ends of the circularizable molecule, and wherein step b) comprises contacting the population of cleaved flaps with the preparation of circularizable molecules and a population of bridge oligonucleotides, wherein each bridge oligonucleotide comprises an inner segment that is complementary to a cleaved flap sequence, a 5′ end complementary to the 5′ end of the circularizable molecule, and a 3′ end that is complementary to a 3′ end of the circularizable molecule.

65. The method of embodiment 64, wherein the circularizable molecules each comprise a 5′ blocking group.

66. The method of any one of embodiments 52-65, wherein the circularizable molecules are dumbbell probes, wherein each dumbbell probe comprises a bridge sequence that is complementary to a cleaved flap sequence.

67. The method of any one of embodiments 49-66, further comprising a step of replicating the circular molecules comprising cleaved flap sequences using rolling circle replication, to form a population of flap replicons.

68. The method of any one of embodiments 49-67, wherein the plurality of flap oligonucleotides in a reaction mixture comprises at least 10 unique flap oligonucleotides.

69. The method of embodiment 68, wherein the plurality of flap oligonucleotides in a reaction mixture comprises at least 20 unique flap oligonucleotides.

70. The method of embodiment 68, wherein the plurality of flap oligonucleotides in a reaction mixture comprises at least 30 unique flap oligonucleotides.

71. The method of embodiment 68, wherein the plurality of flap oligonucleotides in a reaction mixture comprises 40 to 1000 unique flap oligonucleotides.

72. The method of any one of embodiments 49-71, wherein the target-specific portions of flap oligonucleotides in the plurality of flap oligonucleotides in the reaction mixture are at least 6 nucleotides in length, preferably at least 7, preferably at least 8, preferably at least 9, preferably at least 10, preferably at least 11, preferably at least 12 nucleotides in length.

73. The method of embodiment 72, wherein the target-specific portions of the flap oligonucleotides in the plurality of flap oligonucleotides in the reaction mixture are 13 to 100 nucleotides in length.

74. The method of any one of embodiments 49-73, wherein the target nucleic acid comprises one or more of cDNA, amplified DNA, genomic DNA, and circulating cell-free DNA.

75. The method of any one of embodiments 49-74, wherein the target nucleic acid comprises DNA treated with a methylation-specific reagent.

76. The method of embodiment 75, wherein the reaction mixture further comprises oligonucleotides specific for a reference nucleic acid.

77. The method of embodiment 76, wherein the oligonucleotides comprise a flap oligonucleotide, an invasive oligonucleotide, and optionally a non-invasive oligonucleotide.

78. The method of embodiment 76 or embodiment 77, wherein a concentration of one or more of the oligonucleotides specific for the reference nucleic acid is altered in the reaction mixture relative to a concentration of corresponding oligonucleotides specific for the target nucleic acids in the reaction mixture and/or wherein one or more of the oligonucleotides specific for the reference nucleic acid binds to the reference nucleic acid with one or more mismatched base pairings.

79. The method of embodiment 78, wherein the concentration of the one or more of the oligonucleotides specific for the reference nucleic acid is reduced in the reaction mixture relative to the concentration of the corresponding oligonucleotides specific for the target nucleic acids in the reaction mixture.

80. The method of any one of embodiments 76 to 79, wherein the reference nucleic acid comprises methylated *B3GALT6* DNA.

81. A composition comprising:

i) one or both of:

a) a population of cleaved flaps; and

b) a population of circular molecules, each circular molecule comprising a cleaved flap sequence or the complement thereof, wherein each cleaved flap sequence or complement thereof comprises a sequence of nucleotides indicative of a target nucleic acid;

and

ii) a ligase enzyme.

82. The composition of embodiment 81, further comprising a population of bridge oligonucleotides, wherein each bridge oligonucleotide comprises a 5′ end sequence that is complementary to a 5′ end of a cleaved flap sequence, and a 3′ end sequence that is complementary a 3′ end of the cleaved flap sequence, wherein hybridization of the bridge oligonucleotide to a cleaved flap, if present in the composition, forms a ligatable nick.

83. The composition of embodiment 81, further comprising circularizable molecules, wherein each circular molecule comprises a circularizable molecule sequence.

84. The composition of embodiment 81 or embodiment 83, further comprising a population of bridge oligonucleotides, wherein each bridge oligonucleotide comprises an inner segment that is complementary to a cleaved flap sequence, a 3′ end complementary to the 3′ end of a circularizable molecule, and a 5′ end complementary to a 5′ end of the circularizable molecule.

85. The composition of embodiment 84, further comprising one or more of the following:

i) a circularizable vector molecule comprising a 5′ blocking group, wherein in the composition, the bridge oligonucleotide is hybridized to the cleaved flap and to and to the circularizable molecule to form a complex comprising:

I) a first ligatable nick comprising the 3′ end of the circularizable molecule and a 5′ end of the cleaved flap, and

II) an invasive cleavage substrate comprising a 3′ end of the cleaved flap and the 5′ end of the circularizable molecule comprising the 5′ blocking group;

wherein the 5′ blocking group preferably comprises a moiety selected from a hexane, a non-standard nucleotide, an abasic nucleotide, a fluorophore, a flap nucleic acid sequence, a hairpin flap nucleic acid sequence, and a hydroxyl group;

ii) a bridge oligonucleotide comprising one or more of an RNA portion and a DNA portion;

iii) a bridge oligonucleotide comprising an RNA portion comprising one or more nucleotide modifications, preferably selected from 2′-O-methyl and 2′-O-methoxyethyl nucleotide modifications;

iv) a flap endonuclease, preferably a FEN-1 endonuclease, preferably a strict FEN-1 endonuclease, preferably a strict FEN-1 endonuclease selected from the group consisting of *Archaeoglobus fulgidus* (Afu) FEN-1, *Pyrococcus furiosus* (Pfu) FEN-1, and *Archaeoglobus veneficus* (Ave) FEN-1, and variants thereof, and CLEAVASE 2.0 nuclease;

v) a Mg^{++} flap assay buffer;

vi) amplification reagents;

vii) flap assay reagents; and/or

viii) a ligase comprises *Chlorella* virus PBCV-1 DNA ligase.

86. The composition of any one of embodiments 81-85, wherein each circular molecule in the population of circular molecules comprises one or more of:

i) a pair of amplification primer binding sites configured to amplify a portion of a circular molecule that comprises a cleaved flap sequence;

ii) a primer binding site configured to amplify a circular molecule by rolling circle amplification;

iii) at least one unique molecular tag;

iv) a polynucleotide linker; and/or

v) a polymerization-terminating molecule, preferably an internal spacer.

87. The composition of any one of embodiments 81-86, further comprising a population of amplified products, each amplified product comprising a target nucleic acid sequence that is associated with a cleaved flap sequence in a circular molecule in the population of circular molecules.

88. A method, comprising:

a) providing in a mixture a bridge oligonucleotide, a flap sequence oligonucleotide, preferably a cleaved flap oligonucleotide, and a circularizable molecule comprising a 5′ blocking group, wherein the bridge oligonucleotide comprises:

i) an inner segment that is complementary to the flap sequence oligonucleotide,

ii) a 3′ end that is complementary to a 3′ end of the circularizable molecule, and

iii) a 5′ end that is complementary to a 5′ end of the circularizable molecule,

wherein in the mixture, the bridge oligonucleotide is hybridized to the flap sequence oligonucleotide and to the circularizable molecule to form a complex comprising:

I) a first ligatable nick comprising the 3′ end of the circularizable molecule and a 5′ end of the flap sequence oligonucleotide, and

II) an invasive cleavage substrate comprising a 3′ end of the flap sequence oligonucleotide and the 5′ end of the circularizable molecule comprising the 5′ blocking group;

b) treating the complex with a flap endonuclease in conditions wherein cleavage of the invasive cleavage substrate cleaves off the 5′ blocking group and produces a second ligatable nick; and

c) treating the complex with a ligase under conditions wherein the first ligatable nick and the second ligatable nick are ligated to form a circular molecule.

89. The method of embodiment 88, wherein the 5′ blocking group comprises a moiety selected from a hexane, a non-standard nucleotide, an abasic nucleotide, a fluorophore, a flap nucleic acid sequence, a hairpin flap nucleic acid sequence, and a hydroxyl group.

90. The method of embodiment 88 or 89, wherein the bridge oligonucleotide comprises one or more of an RNA portion and a DNA portion.

91. The method of embodiment 90, wherein the RNA portion comprises one or more nucleotide modifications.

92. The method of embodiment 91, wherein the one or more nucleotide modifications comprise 2′-O-methyl or 2′-O-methoxyethyl modifications.

93. The method of embodiment 91 or embodiment 92, wherein the 5′ end and an adjacent part of the inner segment of the bridge oligonucleotide comprise RNA, 2′-O-methyl RNA, or 2′-O-methoxyethyl RNA.

94. The method of embodiment 93, wherein the 3′ end and the adjacent part of the inner segment of the bridge oligonucleotide comprise DNA.

95. The method of any one of embodiments 93-94, further comprising detecting the circular molecule in a nucleic acid detection assay.

96. The method of any one of embodiments 88-95, wherein the ligase comprises *Chlorella* virus PBCV-1 DNA ligase.

97. A method comprising:

a) providing a reaction mixture comprising or suspected of comprising at least one target nucleic acid, at least one flap oligonucleotide, and a flap endonuclease, wherein each flap oligonucleotide comprises:

i) a target-specific portion that anneals to a target nucleic acid, if the target nucleic acid is present in the reaction mixture, and

ii) a 5′ flap portion, comprising a sequence of nucleotides indicative of the target nucleic acid to which the target-specific portion anneals;

wherein, for target nucleic acids present in the reaction mixture, invasive cleavage structures comprising a target nucleic acid annealed to a flap oligonucleotide are formed;

wherein, in the reaction mixture, invasive cleavage structures are cleaved by the flap endonuclease to produce at least one cleaved flap;

b) providing at least one ligated molecule, wherein the ligated molecule comprises sequence of the cleaved flap, and/or a sequence complementary to the cleaved flap, and

c) characterizing the at least one ligated molecule to characterize the at least one target nucleic acid present in the reaction mixture.

98. The method of embodiment 97, wherein the ligated molecule is a circular molecule.

99. The method of embodiment 97, wherein the ligated molecule is a linear molecule.

100. The method of embodiment 97, wherein providing the ligated molecule comprises treating the cleaved flap with a preparation comprising a ligatable vector molecule under conditions wherein a cleaved flap is ligated to the ligatable vector molecule to form a ligated molecule, each ligated molecule comprising a vector molecule sequence and a cleaved flap sequence.

101. The method of any one of embodiments 97-100, wherein the reaction mixture is an amplification reaction mixture, and wherein the at least one target nucleic acid comprises at least one amplified nucleic acid produced in the amplification reaction mixture.

102. The method of embodiment 101, wherein the amplification reaction mixture is a PCR-flap assay reaction mixture.

103. The method of any one of embodiments 97-102, wherein characterizing the at least one ligated molecule comprises counting different cleaved flap sequences, or complements thereof, if present in the reaction mixture.

104. The method of embodiment 103, wherein characterizing the at least one ligated molecule comprises sequencing cleaved flap sequences, or the complements thereof, if present in the reaction mixture.

105. The method of any one of embodiments 97-104, wherein the flap endonuclease is a FEN-1 endonuclease.

106. The method of embodiment 105, wherein the FEN-1 endonuclease is a strict FEN-1 endonuclease, and wherein the reaction mixture comprises a Mg^{++} flap assay buffer.

107. The method of embodiment 106, wherein the strict FEN-1 endonuclease is selected from the group consisting of *Archaeoglobus fulgidus* (Afu) FEN-1, *Pyrococcus furiosus* (Pfu) FEN-1, and *Archaeoglobus veneficus* (Ave) FEN-1, and variants thereof, and CLEAVASE 2.0 nuclease.

108. The method of any one of embodiments 97-107, wherein each ligated molecule comprises one or more of:

i) a first bridge oligonucleotide-complementary arm at a 3′ end of a first vector sequence and a second bridge oligonucleotide-complementary arm at a 5′ end of a second vector sequence, wherein the first bridge oligonucleotide-complementary arm has a different nucleotide sequence than the second bridge oligonucleotide-complementary arm;

ii) a pair of amplification primer binding sites configured to amplify a portion of a ligated molecule that comprises a cleaved flap sequence;

iii) a primer binding site configured to amplify a circular ligated molecule by rolling circle amplification;

iv) at least one unique molecular tag;

v) a polynucleotide linker; and/or

vi) a polymerization-terminating molecule, preferably an internal spacer.

109. The method of any one of embodiments 97-108, further comprising a step of amplifying a region of the at least one ligated molecule comprising a cleaved flap sequence to form at least one flap amplicon.

110. The method of embodiment 109, further comprising sequencing the at least one flap amplicon to produce sequencing reads.

111. The method of any one of embodiments 100-110, wherein the ligatable vector molecule is selected from a dumbbell probe, a padlock probe, a vector pair, and/or a linear vector molecule.

112. The method of any one of embodiments 100-111, wherein the ligatable vector molecule comprises a vector pair comprising a first bridge oligonucleotide-complementary arm at a 3′ end of a first vector sequence and a second bridge oligonucleotide-complementary arm at a 5′ end of a second vector sequence, and wherein step b) comprises contacting the at least one cleaved flap with the ligatable vector molecule and a bridge oligonucleotide, wherein the bridge oligonucleotide comprises an inner segment that is complementary to a cleaved flap sequence, a 3′ end that is complementary to the 3′ end of the first vector sequence, and a 5′ end that is complementary to the 5′ end of the second vector sequence.

113. The method of embodiment 112, wherein the ligatable vector molecule comprising the 5′ end of the second vector sequence comprises a 5′ blocking group.

114. The method of any one of embodiments 100-111, wherein the ligatable vector molecule is a dumbbell probe comprising a bridge sequence that is complementary to a cleaved flap sequence.

115. The method of any one of embodiments 97-111 and 114, wherein the ligated molecule is a circular molecule, the method further comprising a step of replicating the circular molecule comprising the cleaved flap sequence using rolling circle replication, to form a flap replicon.

116. The method of any one of embodiments 97-115, wherein the target-specific portions of the flap oligonucleotide in the reaction mixture is at least 6 nucleotides in length, preferably at least 7, preferably at least 8, preferably at least 9, preferably at least 10, preferably at least 11, preferably at least 12 nucleotides in length.

117. The method of embodiment 116, wherein the target-specific portion of the flap oligonucleotide in the reaction mixture is 13 to 100 nucleotides in length.

118. The method of any one of embodiments 97-117, wherein the target nucleic acid comprises one or more of cDNA, amplified DNA, genomic DNA, and circulating cell-free DNA.

119. The method of any one of embodiments 97-118, wherein the target nucleic acid comprises DNA treated with a methylation-specific reagent.

120. The method of embodiment 119, wherein the reaction mixture further comprises oligonucleotides specific for a reference nucleic acid, the reference nucleic acid preferably comprising methylated *B3GALT6* DNA.

121. The method of embodiment 120, wherein the oligonucleotides comprise a flap oligonucleotide, an invasive oligonucleotide, and optionally a non-invasive oligonucleotide.

122. The method of embodiment 120 or embodiment 121, wherein a concentration of one or more of the oligonucleotides specific for the reference nucleic acid is altered in the reaction mixture relative to a concentration of corresponding oligonucleotides specific for the target nucleic acid in the reaction mixture, and/or wherein one or more of the oligonucleotides specific for the reference nucleic acid binds to the reference nucleic acid with one or more mismatched base pairings.

123. The method of embodiment 122, wherein the concentration of the one or more of the oligonucleotides specific for the reference nucleic acid is reduced in the reaction mixture relative to the concentration of the corresponding oligonucleotides specific for the target nucleic acid in the reaction mixture.

124. A composition comprising:

i) one or both of:

a) at least one cleaved flap; and

b) at least one ligated molecule comprising a cleaved flap sequence or a complement thereof, wherein the cleaved flap sequence or complement thereof comprises a sequence of nucleotides indicative of a target nucleic acid;

and

ii) a ligase enzyme.

125. The composition of embodiment 124, further comprising at least one bridge oligonucleotide, wherein each bridge oligonucleotide comprises a 5′ end sequence that is complementary to a 5′ end of a cleaved flap sequence, and a 3′ end sequence that is complementary a 3′ end of the cleaved flap sequence, wherein hybridization of the bridge oligonucleotide to a cleaved flap, if present in the composition, forms a ligatable nick.

126. The composition of embodiment 124, further comprising at least one ligatable vector molecule, wherein each ligated molecule comprises at least one ligatable vector molecule sequence.

127. The composition of embodiment 124 or embodiment 126, further comprising at least one bridge oligonucleotide, wherein each bridge oligonucleotide comprises an inner segment that is complementary to a cleaved flap sequence, a 3′ end that is complementary to a 3′ end of a first vector sequence, and a 5′ end that is complementary to the 5′ end of a second vector sequence.

128. The composition of embodiment 127, further comprising one or more of the following:

i) a first ligatable vector molecule and second ligatable vector molecule, the second ligatable vector molecule comprising a 5′ blocking group, wherein in the composition, the bridge oligonucleotide is hybridized to the cleaved flap and to the first and second ligatable vector molecules in a complex comprising:

I) a first ligatable nick comprising the 3′ end of the first ligatable molecule and a 5′ end of the cleaved flap, and

II) an invasive cleavage substrate comprising a 3′ end of the cleaved flap and the 5′ end of the second ligatable vector molecule comprising the 5′ blocking group,

wherein the 5′ blocking group preferably comprises a moiety selected from a hexane, a non-standard nucleotide, an abasic nucleotide, a fluorophore, a flap nucleic acid sequence, a hairpin flap nucleic acid sequence, and a hydroxyl group;

ii) a bridge oligonucleotide comprising one or more of an RNA portion and a DNA portion;

iii) a bridge oligonucleotide comprising an RNA portion comprising one or more nucleotide modifications, preferably selected from 2′-O-methyl and 2′-O-methoxyethyl nucleotide modifications;

iv) a flap endonuclease, preferably a FEN-1 endonuclease, preferably a strict FEN-1 endonuclease, preferably a strict FEN-1 endonuclease selected from the group consisting of *Archaeoglobus fulgidus* (Afu) FEN-1, *Pyrococcus furiosus* (Pfu) FEN-1, and *Archaeoglobus veneficus* (Ave) FEN-1, and variants thereof, and CLEAVASE 2.0 nuclease;

v) a Mg^{++} flap assay buffer;

vi) amplification reagents;

vii) flap assay reagents; and/or

viii) a ligase comprises *Chlorella* virus PBCV-1 DNA ligase.

129. The composition of any one of embodiments 124-128, wherein the ligated molecule comprises one or more of:

i) a pair of amplification primer binding sites configured to amplify a portion of a ligated molecule that comprises a cleaved flap sequence;

ii) a primer binding site configured to amplify a circular ligated molecule by rolling circle amplification;

iii) at least one unique molecular tag;

iv) a polynucleotide linker; and/or

v) a polymerization-terminating molecule, preferably an internal spacer.

130. The composition of any one of embodiments 124 to 129, further comprising at least one amplified product, each amplified product comprising a target nucleic acid sequence that is associated with a cleaved flap sequence in a ligated molecule.

131. A method, comprising:

a) providing in a mixture a bridge oligonucleotide, a flap sequence oligonucleotide, preferably a cleaved flap oligonucleotide, a first ligatable vector molecule and second ligatable vector molecule comprising a 5′ blocking group, wherein the bridge oligonucleotide comprises:

i) an inner segment that is complementary to the flap sequence oligonucleotide,

ii) a 3′ end that is complementary to a 3′ end of the first ligatable vector molecule, and

iii) a 5′ end that is complementary to a 5′ end of the second ligatable vector molecule,

wherein, in the mixture, the bridge oligonucleotide is hybridized to the flap sequence oligonucleotide and to the first and second ligatable molecules to form a complex comprising:

I) a first ligatable nick comprising the 3′ end of the first ligatable molecule and a 5′ end of the flap sequence oligonucleotide, and

II) an invasive cleavage substrate comprising a 3′ end of the flap sequence oligonucleotide and the 5′ end of the second ligatable vector molecule comprising the 5′ blocking group;

b) treating the complex with a flap endonuclease in conditions wherein cleavage of the invasive cleavage substrate cleaves off the 5′ blocking group of the second ligatable vector molecule and produces a second ligatable nick; and

c) treating the complex with a ligase under conditions wherein the first ligatable nick and the second ligatable nick are ligated to form a ligated molecule.

132. The method of embodiment 131, wherein the 5′ blocking group comprises a moiety selected from a hexane, a non-standard nucleotide, an abasic nucleotide, a fluorophore, a flap nucleic acid sequence, a hairpin flap nucleic acid sequence, and a hydroxyl group.

133. The method of embodiment 131 or 132, wherein the bridge oligonucleotide comprises one or more of an RNA portion and a DNA portion.

134. The method of embodiment 133, wherein the RNA portion comprises one or more nucleotide modifications.

135. The method of embodiment 134, wherein the one or more nucleotide modifications comprise 2′-O-methyl or 2′-O-methoxyethyl modifications.

136. The method of embodiment 134 or embodiment 133, wherein the 5′ end and an adjacent part of the inner segment of the bridge oligonucleotide comprise RNA, 2′-O-methyl RNA, or 2′-O-methoxyethyl RNA.

137. The method of embodiment 134, wherein the 3′ end and an adjacent part of the inner segment of the bridge oligonucleotide comprise DNA.

138. The method of any one of embodiments 131-137, further comprising detecting the ligated molecule in a nucleic acid detection assay.

DEFINITIONS

To facilitate an understanding of the present technology, a number of terms and phrases are defined below. Additional definitions are set forth throughout the detailed description.

Throughout the specification and claims, the following terms take the meanings explicitly associated herein, unless the context clearly dictates otherwise. The phrase “in one embodiment” as used herein does not necessarily refer to the same embodiment, though it may. Furthermore, the phrase “in another embodiment” as used herein does not necessarily refer to a different embodiment, although it may. Thus, as described below, various embodiments of the technology may be readily combined, without departing from the scope or spirit of the technology.

In addition, as used herein, the term “or” is an inclusive “or” operator and is equivalent to the term “and/or” unless the context clearly dictates otherwise. The term “based on” is not exclusive and allows for being based on additional factors not described unless the context clearly dictates otherwise. In addition, throughout the specification, the meaning of “a,” “an,” and “the” include plural references. The meaning of “in” includes “in” and “on.”

As used herein, the transitional term "comprising" is synonymous with "including," "containing," and "characterized by," and is inclusive or open-ended and does not exclude additional, unrecited elements or method steps.

The transitional phrase "consisting essentially of" as used in claims in the present application limits the scope of a claim to the specified materials or steps "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention, as discussed in In re Herz, 537 F.2d 549, 551-52, 190 USPQ 461, 463 (CCPA 1976). For example, a composition "consisting essentially of" recited elements may contain an unrecited contaminant at a level such that, though present, the contaminant does not alter the function of the recited composition as compared to a pure composition, *i.e.,* a composition "consisting of" the recited components.

The term "each," as used in the claims of the present application in reference to populations or pluralities of elements that each have a particular recited feature, *e.g.*, "each oligonucleotide," "each flap," "each ligated molecule" in a population oligonucleotides, flaps, ligated molecules, respectively, is read as pertaining to a group of those elements that have the recited feature, but does not exclude the presence of similar elements that do not have the recited feature. For example, in a mixture having "a population of oligonucleotides that each have a 5′ blocking group," the mixture may also comprise oligonucleotides that do not have a 5′ blocking group.

The term "primer" refers to an oligonucleotide, whether occurring naturally as, *e.g.,* a nucleic acid fragment from a restriction digest, or produced synthetically, that is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product that is complementary to a nucleic acid template strand is induced, (*e.g.,* in the presence of nucleotides and an inducing agent such as a DNA polymerase, and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. Generally, the primer is sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the

primers will depend on many factors, including temperature, source of primer, and the use of the method.

As used herein, the term "nucleic acid detection assay" refers to any method of determining the nucleotide composition of a nucleic acid of interest. Nucleic acid detection assay include but are not limited to, sequencing methods, probe hybridization methods, structure specific cleavage assays (*e.g.,* the "INVADER" flap assay, or invasive cleavage assay, (Hologic, Inc.) described, *e.g.,* in U.S. Patent Nos. 5,846,717, 5,985,557, 5,994,069, 6,001,567, 6,090,543, and 6,872,816; Lyamichev *et al*., Nat. Biotech., 17:292 (1999), Hall *et al*., PNAS, USA, 97:8272 (2000), and in combined PCR/invasive cleavage assays (Hologic, Inc., *e.g.,* in U.S. Patent Publications 2006/0147955 and 2009/0253142), each of which is herein incorporated by reference in its entirety for all purposes); enzyme mismatch cleavage methods (*e.g.,* Variagenics, U.S. Pat. Nos. 6,110,684, 5,958,692, 5,851,770, herein incorporated by reference in their entireties); polymerase chain reaction (PCR), described above; branched hybridization methods (*e.g.,* Chiron, U.S. Pat. Nos. 5,849,481, 5,710,264, 5,124,246, and 5,624,802, herein incorporated by reference in their entireties); rolling circle replication (*e.g.,* U.S. Pat. Nos. 6,210,884, 6,183,960 and 6,235,502, herein incorporated by reference in their entireties); the variation of rolling circle amplification called "RAM amplification" (see, *e.g.,* US 5,942,391, incorporated herein by reference in its entirety; Fan Li, *et al*., J Clin Microbiol. 2005 Dec; 43(12): 6086–6090.); NASBA (*e.g.,* U.S. Pat. No. 5,409,818, herein incorporated by reference in its entirety); molecular beacon technology (*e.g.,* U.S. Pat. No. 6,150,097, herein incorporated by reference in its entirety); E-sensor technology (U.S. Pat. Nos. 6,248,229, 6,221,583, 6,013,170, and 6,063,573, herein incorporated by reference in their entireties); cycling probe technology (*e.g.,* U.S. Pat. Nos. 5,403,711, 5,011,769, and 5,660,988, herein incorporated by reference in their entireties); Dade Behring signal amplification methods (*e.g.,* U.S. Pat. Nos. 6,121,001, 6,110,677, 5,914,230, 5,882,867, and 5,792,614, herein incorporated by reference in their entireties); ligase chain reaction (*e.g.,* Barany Proc. Natl. Acad. Sci USA 88, 189-93 (1991)); and sandwich hybridization methods (*e.g.,* U.S. Pat. No. 5,288,609, herein incorporated by reference in its entirety).

As used herein in reference to the analysis of nucleic acids, the terms "sequencing," "DNA sequencing," and "RNA sequencing" refer broadly to any method of determining an order

of nucleotides or nucleobases in a strand of nucleic acid or a region of a strand of nucleic acid. Sequencing methods include but are not limited to methods using chemicals or enzymes to break nucleic acid strands in a nucleobase-specific manner (*e.g.,* "Maxam-Gilbert" sequencing," Maxam AM, Gilbert W (February 1977). *Proc. Natl. Acad. Sci. USA. **74** (2): 560–64*); methods using templated primer extension in the presence of chain-terminating reagents, *e.g.,* dideoxynucleotides, as used in "Sanger" sequencing (Sanger F, *et al. Proc. Natl. Acad. Sci. USA. **74** (12): 5463–77 (1977)*; Smith LM, *et al., Nature. **321** (6071): 674–679* (1986*);* and cycle sequencing (see, *e.g.,* Murray V. *Nucleic Acids Res*. 17(21):8889 (1989)) and methods in which each nucleotide added to an extending chain by a polymerase is monitored as it is added ("sequencing-by-synthesis" or "SBS" ; see, *e.g.,* Nyren P, *et al*., *Analytical Biochemistry. **208** (1): 171 (1993*), each of which is incorporated by reference herein in its entirety, for all purposes.

The terms "next generation sequencing" and "NGS" refer to any parallelized method of performing nucleic acid sequencing, preferably highly parallelized methods, including but not limited to sequencing-by-synthesis and sequencing-by-ligation platforms. NGS sequencing platforms include, but are not limited to, the following: Massively Parallel Signature Sequencing (Lynx Therapeutics); 454 pyrosequencing (454 Life Sciences/Roche Diagnostics); solid-phase, reversible dye-terminator sequencing (Solexa/Illumina™); SOLiD™ technology (Applied Biosystems); Ion semiconductor sequencing (Ion Torrent™); and DNA nanoball sequencing (Complete Genomics, BGI Americas). Descriptions of certain NGS platforms can be found in the following: Shendure, er al., "Next-generation DNA sequencing," Nature, 2008, vol. 26, No. 10, 135-1 145; Mardis, "The impact of next-generation sequencing technology on genetics," Trends in Genetics, 2007, vol. 24, No. 3, pp. 133-141; Su, *et al*., "Next-generation sequencing and its applications in molecular diagnostics" Expert Rev Mol Diagn, 2011, 11 (3):333-43; and Zhang *et al*., "The impact of next-generation sequencing on genomics", J Genet Genomics, 201, 38(3): 95-109. Nanopore sequencing methods, such as are offered by Oxford Nanopore Technologies, are described, *e.g.,* by Clarke J, *et al*., *Nature Nanotechnology.* **4**(4): 265–70 (2009); and in US Pat. Nos. 5,795,782, 2, 6,015,714, 6,627,067, 7,238,485 and 7,258,838; The Ion Torrent technology commercialized by Life Technologies is further described, *e.g.,* by Rusk N, *Nat Methods.* **8**(1): 44 (2011); and Dewey FE, *et al., Circulation.* **125** (7): 931–944 (2012). Further description of high-throughput sequencing methods for combinatorial indexing from

amplified nucleic acids as produced in embodiments of the present technology, is provided in US 2023/0193356. As used herein, the term "nanopore" as used in reference to polymer analysis refers to a nano-scale aperture, *e.g.,* in a membrane, configured for passage of a single strand of a polymer (*e.g.,* DNA or RNA), in a manner permitting analysis of each individual monomer unit in the strand as the polymer passes through the pore. Accordingly, "nanopore sequencing" as used in reference to analysis of nucleic acid polymers refers to detecting a sequence of bases in a nucleic acid strand by passage of a strand of nucleic acid through a nanopore. See, *e.g.,* Soni *et al.*, 2007 *Clin Chem* 53: 1996-2001, incorporated herein by reference in its entirety. In typical embodiments, an electric field or a molecular motor is used to drive nucleic acid strands through specialized nanopores configured to detect characteristic changes in current at the nanopore that are characteristic of different nucleotides in a polynucleotide strand. See, *e.g.,* U.S., Patent Nos. 6,015,714 to Baldarelli, *et al*. Issued Jan. 18, 2000, and 8,673,556 to Akeson, *et al*., issued March 18, 2014, and Y. Feng, *et al*., Nanopore-based Fourth-generation DNA Sequencing Technology, *Genomics, Proteomics & Bioinformatics*, **13** (1): 4-16 (2015), each of which is incorporated herein in its entirety.

A used herein, the term "nanopore device" refers to a device suitable for characterizing monomer subunits of a polymer molecule, *e.g.,* nucleotides in a polynucleotide, by a method that comprises passing the polynucleotide through a nanopore. Nanopore devices include but are not limited to the MinION® real-time sequencer from Oxford Nanopore Technologies, Inc.

As used herein, "rolling circle amplification" refers to *in vitro* rolling circle replication of a circular nucleic acid using a strand-displacing DNA polymerase to form a DNA molecule comprising tandem repeats of a sequence complementary to the circular nucleic acid, as described, *e.g.,* in U.S. Pat. Nos. 6,210,884; 6,183,960; 6,235,502; 5,942,391; 6,316,229; 7,862,999;; and in M. Ali, *et al.* "Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine". *Chemical Society Reviews*. **43** (10): 3324–3341 (2014). Rolling circular amplification can be performed using a suitable circularizable molecule as the template, which interacts with a target (if present) to form a structure that can be amplified by rolling circle amplification.

As used herein, the "rolling circle transcription" or "RCT" refers to *in vitro* rolling circle transcription from a circular nucleic acid using an RNA polymerase to form an RNA molecule comprising tandem repeats of a sequence complementary to the circular nucleic acid, as described, *e.g.,* by SL Daubendiek, *et al*. *Rolling Circle RNA Synthesis: Circular Oligonucleotides as Efficient Substrates for T7 RNA Polymerase*. J Am Chem Soc. 1995;117:7818–7819. Circularized oligonucleotides do not require a canonical promoter sequence to serve as templates for rolling circle transcription.

The term "circularizable probe" is also referred to herein as a "circularizable molecule" and is used herein in the broadest sense and refers to any oligonucleotide or probe that can form or be made to form a circular structure, *e.g.,* a covalently closed circular strand of nucleic acid, in the presence of a target nucleic acid or in the presence of a product, *e.g.*, a cleaved flap, that is indicative of the presence of a target nucleic acid. In some embodiments, a circularizable molecule is a padlock probe, which forms a circular structure under the appropriate conditions in the presence of a target (*e.g.,* a cleaved flap sequence) and a ligase. In some embodiments, a circularizable molecule is a dumbbell probe, which forms a closed loop-stem-loop structure (*e.g.,* a circular structure comprising two stem-loops joined by a stem duplex) under the appropriate conditions in the presence of a target (*e.g.,* a cleaved flap sequence) and a ligase. In some embodiments, a circularizable molecule is configured to comprise a feature that restricts ligation, preferably a feature that is removable or repairable in the presence of a target nucleic acid, *e.g.*, a cleaved flap. For example, in some embodiments, when a circularizable molecule is combined with a cleaved flap, one or both of the circularizable molecule or the cleaved flap must be modified, *e.g.*, with a nucleic acid-modifying enzyme such as a polymerase or nuclease, to form the ligatable nick(s).

As used herein, the term "circular molecule" as used in reference to nucleic acid refers to a single- or double-stranded nucleic acid wherein at least one strand is in the form of a covalently closed circle that lacks free 3′ and 5′ termini. Embodiments of circular molecules include oligonucleotides synthesized to be in circular form prior to use ("pre-circularized"), *e.g.*, for use as template for rolling circle replication, *e.g.*, as illustrated in Fig. 3. In some embodiments, circularizable probes are used in their linear form, and are ligated to form circularized molecules

as a step in a detection assay, *e.g.*, as illustrated in Fig. 6A. A circular molecule may be pre-circularized or may become circularized as part of a reaction or assay.

As used herein, the term "linear" as used in reference to a nucleic acid molecule refers to a strand of nucleic acid that has 5′ and 3′ termini that are not covalently attached to each other, such that the strand of nucleic acid does not form a circle.

The term "stem-loop" as used in reference to nucleic acid refers to an intra-polynucleotide structure formed by hybridization of two regions of the same nucleic acid strand to form a duplexed stem that has at one end an unpaired region, the loop, connecting the two duplexed regions. Loops typically comprise at least 3 nucleotides.

The term "dumbbell probe" as used herein refers to a probe comprising stem-loops, preferably forming a loop-stem-loop structure upon hybridization to a complementary nucleic acid, *e.g.,* a cleaved flap. In some embodiments a dumbbell probe is provided as a pre-formed circular molecule, suitable for use as a template, *e.g.,* in an RCA reaction primed by a cleaved flap molecule. In some embodiments, a circularizable dumbbell probe forms a circular structure only under the appropriate conditions (appropriate ligation conditions) and in the presence of a target (*e.g.,* a cleaved flap sequence). In some embodiments, a dumbbell probe comprises a bridge sequence between two stem-loops that is complementary to a flap sequence of a flap oligonucleotide. In some such embodiments, hybridization of the dumbbell probe to the flap portion of an uncleaved flap oligonucleotide forms a complex that cannot be ligated to form a circular molecule (see, *e.g.,* FIG. 5B, bottom right). When the flap oligonucleotide is cleaved, *e.g.,* when an invasive cleavage structure comprising the probe is treated with a flap endonuclease, hybridization of the dumbbell probe to the resulting cleaved flap sequence forms a complex that can be ligated to form a circular molecule (see, *e.g.,* FIG. 5B, bottom left). In some embodiments, the stem of a dumbbell probe comprises a bridge sequence comprising 10-20 unpaired nucleotides that are complementary to a flap sequence or complementary to a cleaved flap sequence. In some embodiments, the stem comprises additional bases 5′ and/or 3′ to the bridge sequence that are not complementary to the flap sequence or the cleaved flap sequence. In some embodiments, the dumbbell probe additionally comprises a loop comprising any suitable number of nucleotides, wherein the base sequence in the loop is not substantially complementary

to the flap sequence or the cleaved flap sequence. In preferred embodiments, the dumbbell probe comprises two stem-loops separated by a bridge sequence.

As used herein, the term "vector pair" refers to a vector or flap vector that comprises a pair of molecules, wherein a first vector oligonucleotide comprises a first bridge-complementary region that is complementary to the 5′ end sequence or terminus of a bridge oligonucleotide and a second vector oligonucleotide comprises a second bridge-complementary region that is complementary to the 3′ end or terminus of the bridge oligonucleotide. In preferred embodiments, a vector pair hybridizes to a bridge oligonucleotide such that a third oligonucleotide, *e.g.*, a cleaved flap, may be hybridized to an inner segment of the bridge oligonucleotide such that the third oligonucleotide is positioned between the two oligonucleotides of the vector pair, preferable forming ligatable nicks, or nicks that may be made ligatable by an additional step, *e.g.*, cleavage of a 5′ flap from one of the oligonucleotides of the vector pair.

In some embodiments, target nucleic acid is amplified (*e.g.,* by PCR) and amplified nucleic acid is detected using an invasive cleavage assay to produce cleaved flap sequences. In some embodiments, the target nucleic acid is amplified (*e.g.,* by PCR) and an invasive cleavage assay is performed simultaneously. In some embodiments, the target nucleic acid is amplified (*e.g.,* by PCR) and an invasive cleavage assay is performed to produce cleaved flap sequences, and the invasive cleavage assay is followed by further amplification and/or sequencing steps. Assays configured for performing a detection assay (*e.g.,* invasive cleavage assay) in combination with an amplification assay are described in US Patent No. 9,096,893 B2 (App. Ser. No. 13/941,122), incorporated herein by reference in its entirety for all purposes. See also Allawi HT, *et al. J of Clin Microbio* 2006; vol 44, no. 9: 3443-3447. Additional amplification plus invasive cleavage detection configurations, termed the QuARTS method, are described in U.S. Pat. No. 8,361,720, 8,715,937, and 8,916,344, incorporated herein by reference in their entireties for all purposes.

In some embodiments, the target nucleic acid is amplified (*e.g.,* by PCR), and amplified nucleic acid is detected using an invasive cleavage assay, wherein cleavage of an amplified target in an invasive cleavage structure results in the formation of a cleaved flap sequence, which

is then detected, for instance by using a ligatable vector (*e.g.,* a padlock probe, a dumbbell probe, one or a pair of oligonucleotides of a vector pair) that hybridizes and/or ligates to the cleaved flap sequence. In some embodiments, ligation of the cleaved flap sequence to the ligatable vector (*e.g.,* padlock probe, dumbbell probe) results in the formation of a closed circular structure or a linear structure comprising vector ends, the presence of which is further amplified and/or detected (*e.g.,* by using the closed circular structure or linear ligated product as template for PCR, RCA, RCT, sequencing, *etc.*)

As used herein, the terms "invasive cleavage structure" and "overlap cleavage structure" are used interchangeably and refer to a cleavage structure generally comprising a template nucleic acid (*e.g.,* target nucleic acid), an upstream nucleic acid (*e.g.,* an invasive oligonucleotide, or a 3′ portion of a template strand folded back and hybridized to form a hairpin, or a 3′ portion of a padlock probe), and iii) a downstream nucleic acid (*e.g.,* a probe, a target-specific region of a flap oligonucleotide, or a 5′ portion of a template strand folded back and hybridized to form a hairpin, or a 5′ portion of a padlock probe), where the upstream and downstream nucleic acids anneal to adjacent regions of the template strand (*e.g.,* the target nucleic acid) (*i.e.,* regions of a template strand that are next to each other on the strand, not separated by intervening nucleotides or base pairs) to form an upstream duplex and a downstream duplex, as illustrated in FIG.1, and where an overlap forms between the 3′ end of the upstream nucleic acid and the duplex formed between the downstream nucleic acid and the template nucleic acid. In some embodiments, an "invasive cleavage structure" refers to a cleavage structure as shown in FIG. 1, comprising the flap oligonucleotide, a target nucleic acid, and an upstream invasive oligonucleotide, wherein the upstream invasive oligonucleotide forms the upstream duplex with the target nucleic acid, and the target-specific region of the flap oligonucleotide forms the downstream duplex with the target nucleic acid. The flap oligonucleotide also contains the flap sequence, which is cleaved off in the presence of a suitable structure-specific endonuclease.

In some embodiments, an "invasive cleavage structure" refers to a cleavage structure as shown in FIG. 2, comprising the flap oligonucleotide and a target nucleic acid, wherein a 3′ portion of the flap oligonucleotide comprises an invasive portion that hybridizes to a portion of the target nucleic acid to form the upstream duplex and the overlapping 3′ end.

In certain embodiments, the 3′ portion of the upstream nucleic acid, *e.g.,* the invasive oligonucleotide, that overlaps with the downstream duplex is a single nucleotide. In some embodiments, the 3′ portion of the upstream nucleic acid that overlaps with the downstream duplex is a non-base chemical moiety such as an aromatic ring structure, *e.g.,* as disclosed, for example, in U.S. Pat. No. 6,090,543, incorporated herein by reference in its entirety.

As used herein in reference to an invasive cleavage structure, the term "upstream duplex" refers to a duplex formed between a template strand and a hybridized upstream nucleic acid strand having a 3′ end, and the term "downstream duplex" refers to a duplex formed between a template strand and a hybridized downstream nucleic acid having a 5′ end, such that an overlapping flap endonuclease substrate is formed by the adjacent hybridized regions of the upstream and downstream nucleic acid strands. In some embodiments, the upstream nucleic acid and the downstream nucleic acid are part of the same strand of a polynucleotide, such that the invasive cleavage structure comprises 2 nucleic acid strands. See, *e.g.,* FIG. 2, which illustrates the upstream and downstream duplex regions of invasive cleavage structures formed from 2 strands of nucleic acid. In some embodiments, one or more of the nucleic acid strands of an invasive cleavage structure may be attached to each other through a non-nucleic acid chemical linkage (*e.g.,* a multi-carbon chain). See, *e.g.,* U.S. Pat. No. 8,445,238, which is incorporated herein by reference in its entirety. In some embodiments, two nucleic acid strands are attached to each other through a nucleic acid backbone region, forming a padlock or dumbbell probe. Preferably, a nucleic acid backbone in a padlock dumbbell probe is substantially non-complementary to the target nucleic acid.

A "flap oligonucleotide" refers to an oligonucleotide cleavable in a detection assay, such as an invasive cleavage assay, by a flap endonuclease. In some embodiments, a flap oligonucleotide comprises at least one target-specific region (also referred to herein as a target-specific sequence) and a flap sequence (also referred to herein as a flap portion), preferably a 5′ flap portion or sequence. The at least one target-specific region is complementary to a target of interest (*e.g.,* a target nucleic acid), whereas the flap portion is an arm (*e.g.,* a 5′ arm) that is preferably not substantially complementary to the target of interest (*e.g.,* does not bind to the target of interest under assay conditions in which the flap oligonucleotide is used). The flap sequence is not limited to any particular length or sequence. In preferred embodiments, the 5′

flap portion of a flap oligonucleotide is selected such that secondary structure in the flap portion of a flap oligonucleotide does not reduce efficiency of cleavage of the flap oligonucleotide. See, *e.g.*, C. Spiro, *et al*., Molecular Cell Volume 4, Issue 6, December 1999, Pages 1079-1085. Thus, in certain embodiments, the flap sequence is selected to have a sequence that does not form significant secondary structures under the conditions of a cleavage reaction. In some embodiments, the length and/or sequence of the flap sequence is selected to reduce such secondary structure. In some embodiments, the flap sequence is preferably less than about 200 nucleotides, more preferably less than about 175 nucleotides in length. In some embodiments, the flap sequence is from 1 to about 150 nucleotides, preferably 1 to about 100 nucleotides, preferably about 5 to 75 nucleotides. In preferred embodiments, the 5′ flap portion is at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, or 75 nucleotides in length. In certain embodiments, the 5′ flap portion is preferably 6 to 45 nucleotides, preferably 7 to 40 nucleotides, preferably 8 to 35 nucleotides, preferably 8 to 30 nucleotides, preferably 8 to 25 nucleotides, preferably 9 to 25 nucleotides, preferably 10 to 20 nucleotides, preferably 10 to 15 nucleotides in length.

Cleavage of the flap oligonucleotide (*e.g.,* by a flap endonuclease) typically generates a cleaved flap sequence, also referred to herein as a “cleaved flap”, a “cleaved flap segment”, a “cleaved flap portion,” a “cleaved arm”, a “cleaved marker-specific identifier arm”, a “cleaved target-specific marker” or a “cleaved target specific-identifier”. In preferred embodiments, a flap oligonucleotide forms an invasive cleavage structure with one or more other nucleic acids, *e.g.,* a target or template nucleic acid and an invasive oligonucleotide. For example, in some embodiments a flap oligonucleotide forms an invasive cleavage structure with a target nucleic acid strand, wherein the upstream duplex comprises an invasive oligonucleotide and wherein the downstream duplex comprises the target-specific region of a flap oligonucleotide. Such an embodiment is shown schematically in FIG. 1. In some embodiments, the flap oligonucleotide comprises two target-specific regions, and thus does not require a separate invasive oligonucleotide in order to form the invasive cleavage structure. For example, in some embodiments (including the embodiment shown in FIG. 2), the flap oligonucleotide comprises a

first target-specific region (or "flap portion") and a second target-specific region (or "invasive portion" that each bind to different but adjacent regions on the target nucleic acid, thereby forming the upstream and the downstream duplexes in an invasive cleavage structure. In preferred embodiments, the cleaved flap comprises or is composed of the 5′ flap sequence and the nucleotide that is 5′ of the flap endonuclease cleavage site, *e.g.,* as illustrated in FIG. 2.

As used herein, the term "flap endonuclease" or "FEN" refers to a structure-specific nucleolytic enzyme that cleaves a nucleic acid flap structure, *e.g.,* an invasive cleavage structure. Flap endonucleases include, *e.g.,* 5′ -exonuclease domains of the DNA polymerase I proteins of Eubacteria and the FEN-1 proteins of Eukarya and Archaea. (Kaiser M.W., *et al*. (1999) J. Biol. Chem., 274:21387), as well as engineered endonucleases described, *e.g.,* in US 7,935,800, WO 2003/073067, WO 02/070755, and US Patent No. US 7,122,364, which are incorporated herein by reference in their entireties for all purposes. Flap endonuclease may cleave additional structures, *e.g.,* pseudo-Y, 5′ overhang, and gap structures. See, *e.g.,* Shen, B., BioEssays 27:717-729 (2005), and Finger, LD., Subcell Biochem. 62:301–326 (2012), each of which is incorporated herein in its entirety. Flap endonucleases are reviewed by Ceska and Savers (Trends Biochem. Sci. 1998 23:331-336) and Liu, *et al.* (Annu. Rev. Biochem. 2004 73: 589-615), and Tsutakawa S, *et al.* (Cell v145:198-211 (2011)), each herein incorporated by reference in its entirety.

The terms "FEN-1" as used herein in reference to an enzyme refers to a non-polymerase flap endonuclease from a eukaryote or archaeal organism, as encoded by a FEN-1 (Flap Structure-Specific Endonuclease 1) gene. See, *e.g.,* Kaiser, *et al*., *supra*, WO 02/070755, and US Patent No. US 7,122,364, which are incorporated by reference herein in their entireties for all purposes. The term "FEN-1 activity" refers to any enzymatic activity of a FEN-1 enzyme. FEN-1 endonucleases also comprise modified FEN-1 proteins, *e.g.,* chimerical proteins comprising portions of FEN-1 enzymes from different organisms, and enzymes comprising one or more mutations (*e.g.,* substitutions, deletions, insertions, *etc.*), as described in WO 02/070755, and US Patent No. US 7,122,364. The term "FEN1" is used interchangeably with "FEN-1" herein. Preferred FEN-1 endonucleases are thermostable endonucleases, preferably from or derived from an archaeal organism.

The term "thermostable" when used in reference to an enzyme, such as a flap endonuclease, indicates that the enzyme is functional or active (*i.e.,* can perform catalysis) at an elevated temperature, *i.e.,* at about 55°C or higher, and that remains functional or active after exposure to highly elevated temperature, *e.g.,* about 90°C or higher.

As used herein, the term “strict FEN-1” refers to a FEN-1 enzyme, generally from an archaeal organism, that, under suitable conditions, will cleave an invasive cleavage structure but will show substantially no activity in cleaving non-invasive structures, *e.g.,* a gapped structure (having a single-stranded region of target or template strand between the upstream and downstream duplexes), Y-structure (having a 5′ flap but no overlap between the upstream and downstream duplexes), pseudo-Y structure (having a 5′ flap and an un-duplexed template strand upstream of the downstream duplex). Further, a strict FEN-1 endonuclease will not cleave an invasive cleavage structure formed on a target strand composed of RNA nucleotides or analogs thereof, *e.g.*, 2′-O-methyl nucleotides, at and around the cleavage site of the structure. See, *e.g.*, US Pat. No. 7,045,289 to Allawi, *et al*. For example, *Afu* FEN-1 and *Pfu* FEN-1 are a strict FEN-1s when Mg^{++} is the predominant or sole divalent cation in a cleavage buffer, *e.g.,* in a flap assay buffer. Examples of solution conditions under which a strict FEN-1 enzyme will exhibit strict, selective cleavage behavior include but are not limited to solutions comprising 10 mM MOPS, pH 7.5, 5 mM $MgSO_4$, 100 mM KCl, (see US 7,045,289) and 10 mM MOPS, 0.3 mM Tris-HCl, pH 8.0, 7.5 mM $MgCl_2$, and 0.8 mM KCl.

As used herein, the term “PCR-flap assay” is used interchangeably with the term “PCR-invasive cleavage assay” and refers to an assay configuration combining PCR target amplification and detection of the amplified DNA by formation of a first overlap cleavage structure comprising amplified target DNA, and a second overlap cleavage structure comprising a cleaved 5′ flap from the first overlap cleavage structure and, optionally, a reporter oligonucleotide. In some embodiments, the reporter oligonucleotide is labeled.

In some embodiments, a reporter oligonucleotide comprises a linear molecule. In some embodiments, successful cleavage of the flap sequence from a flap oligonucleotide and subsequent ligation of the cleaved flap to the reporter molecule results in formation of a suitable template that is further amplified and/or detected, *e.g.,* by PCR, transcription, sequencing, *etc.*

In some embodiments, a reporter oligonucleotide comprises a circularizable molecule . In some embodiments, successful cleavage of the flap sequence from a flap oligonucleotide and subsequent ligation of the cleaved flap to the circularizable molecule results in formation of a suitable template that is further amplified and/or detected, *e.g.,* by PCR, RCA, RCT, sequencing, *etc.*

In some embodiments, the circularizable molecule comprises a padlock probe. In some embodiments, the padlock probe comprises a 5′ end that is ligated to a 3′ end of the cleaved flap and a 3′ end that is ligated to a 5′ end of the cleaved flap.

The terms “bridge” and “splint” are used interchangeably in reference to oligonucleotides that align the ends of ligatable molecules , *e.g.*, of the ends of a cleaved flap, or a cleaved flap and a circularizable vector, or a cleaved flap and a vector pair, into a ligatable conformation.

In some embodiments, a bridge oligonucleotide comprises an inner segment that is complementary to the cleaved flap sequence, a 5′ segment that is complementary to a first target region of the padlock probe, and a 3′ segment that is complementary to a second target region of the padlock probe. In some embodiments, the bridge oligonucleotide hybridizes to the first target region and second target region of the padlock probe, leaving the inner segment of the bridge oligonucleotide available for binding (*e.g.,* hybridization), such as to a flap sequence or to a cleaved flap sequence. In some embodiments, the cleaved flap sequence hybridizes to the bridge oligonucleotide, thereby generating ligatable nicks. Upon ligation by a suitable ligase, a closed circular structure is formed, which can subsequently be amplified and/or detected by, for example, PCR, RCA, RCT, sequencing, *etc.*

In some embodiments, the flap sequence hybridizes to the bridge oligonucleotide regardless of whether it is cleaved or not cleaved from the flap oligonucleotide (*e.g.,* regardless of whether or not it is cleaved from the target-specific region of the flap oligonucleotide). If no cleavage of the flap oligonucleotide occurs, the flap sequence may be able to hybridize to the bridge oligonucleotide, but ligation at one nick is prevented, and no closed circular structure is formed.

In some embodiments, the circularizable molecule comprises a dumbbell probe. In some embodiments, the circularizable molecule comprises a dumbbell probe comprising a bridge

sequence that is complementary to the cleaved flap sequence, thereby hybridizing to the cleaved flap sequence. In some embodiments, the cleaved flap sequence hybridizes to the bridge sequence, thereby generating ligatable nicks. Upon ligation by a suitable ligase, a closed circular structure is formed (*e.g.,* a dumbbell probe comprising two stem-loops at either end is formed), which can subsequently be amplified and/or detected by, for example, PCR, RCA, RCT, sequencing, *etc.* In contrast, if no cleavage occurs, the flap sequence may be able to hybridize to the bridge sequence, but ligation at one nick is prevented, and thus no closed circular structure is formed.

In some embodiments, a cleaved flap acts as a circularizable molecule. For example, in some embodiments, a cleaved flap is treated with a ligase enzyme under conditions wherein the 5′ end of the cleaved flap is ligated to the 3′ end of the cleaved flap to form a circularized molecule.

In certain preferred embodiments, a single-strand ligase ,*e.g.*, a CIRCLIGASE™ ssDNA ligase, is used. CIRCLIGASE™ ligase enzyme is a thermostable ligase that can form a phosphodiester linkage between the 5′ phosphate and the 3′ hydroxy group of the same or different single DNA or RNA strands (LGC Biosearch, Technologies, Teddington, Middlesex, TW11 0LY, UK or Epicentre Biotechnologies, Madison, WI). CIRCLIGASE II™ is ATP-independent. Preferably, the cleaved flap to be circularized is single stranded and is at least about 10 nucleotides, preferably about 15 nucleotides in length. Nucleic acid circles having 13 or more nucleotides have been shown to work as templates for rolling circle replication. (see, *e.g.*, MG Mohsen and ET Kool, Acc Chem Res. 2016 Nov 15; 49(11): 2540–2550, which is incorporated herein by reference in its entirety, for all purposes.

In some embodiments, the cleaved flap sequence hybridizes to the bridge oligonucleotide, and is subsequently ligated to the padlock probe, thereby forming a closed circular structure. Accordingly, the bridge oligonucleotide facilitates hybridization and stabilization of the cleaved flap sequence for subsequent ligation to the padlock probe and generation of a closed circular structure. In some embodiments, the closed circular structure is amplified and/or detected, for instance, by PCR, RCA, RCT, sequencing, *etc.* In contrast, if the target nucleic acid is not present and therefore no cleavage of the flap oligonucleotide occurs

(*e.g.,* no cleaved flap sequence is released), the flap oligonucleotide sequence hybridizes to the bridge oligonucleotide (*e.g.,* the flap sequence hybridizes to the inner segment of the bridge oligonucleotide) but is unable to ligate to the padlock probe, and therefore the closed circular structure is not formed. For example, in some embodiments the flap sequence hybridizes to the inner segment of the bridge oligonucleotide, but the target-specific region of the oligonucleotide (which is still intact with the flap sequence) interferes with successful ligation.

In some embodiments, the cleaved flap sequence hybridizes to the bridge oligonucleotide, and is subsequently ligated to a vector pair, thereby forming a linear ligated structure. Accordingly, the bridge oligonucleotide facilitates hybridization and stabilization of the cleaved flap sequence for subsequent ligation to the vector pair, and generation of a linear ligated molecule. In some embodiments, the linear ligated molecule is amplified and/or detected, for instance, by PCR, transcription, sequencing, *etc.* However, if the target nucleic acid is not present and therefore no cleavage of the flap oligonucleotide occurs (*e.g.,* no cleaved flap sequence is released), the flap oligonucleotide sequence hybridizes to the bridge oligonucleotide (*e.g.,* the flap sequence hybridizes to the inner segment of the bridge oligonucleotide) but is unable to ligate to both members of a vector pair, and therefore a complete linear ligated molecule is not formed. For example, in some embodiments the flap sequence hybridizes to the inner segment of the bridge oligonucleotide, but the target-specific region of the oligonucleotide (which is still intact with the flap sequence) interferes with successful ligation, *e.g.*, as illustrated in Fig. 5C.

The technology for forming linear ligated molecules is not limited to the use of a vector pair. For example, in some embodiments, the cleaved flap sequence hybridizes to the bridge oligonucleotide, and is subsequently ligated to a single vector oligonucleotide, thereby forming a linear structure.

Ligation, *e.g.,* by a DNA ligase, typically requires a 3′ end of a nucleic acid strand having a 3′ OH group and a 5′ end of a nucleic acid strand having a 5′ phosphate group. In some embodiments, a flap oligonucleotide, *e.g.,* a synthetic oligonucleotide, lacks a 5′ phosphate. For example, the phosphoramidite method of synthesizing oligonucleotides typically produces oligonucleotides having 5′ OH groups. Thus, in some embodiments in which the 5′ end a flap

oligonucleotide will participate in a ligation reaction, the flap oligonucleotide is phosphorylated enzymatically, *e.g.,* using a nucleic acid kinase enzyme (*e.g.,* bacteriophage T4 polynucleotide kinase). In some embodiments the flap oligonucleotide is phosphorylated before use in a flap assay reaction, while in other embodiments, cleaved flaps produced in a flap assay reaction are phosphorylated after the flap assay reaction and prior to a ligation reaction.

In an exemplary PCR-flap assay as used herein, the assay reagents comprise a mixture containing DNA polymerase, FEN-1 endonuclease, a primary probe comprising a portion complementary to a target nucleic acid and a 5′ arm (*e.g.,* a flap oligonucleotide), and a ligatable molecule (*e.g.,* a circularizable molecule, a padlock probe, a dumbbell probe, a linear vector, or one or a pair of oligonucleotides of a vector pair). In some embodiments, the target nucleic acid is amplified by PCR and the amplified nucleic acid is detected simultaneously (*i.e.,* detection occurs during the course of target amplification). In some embodiments, the target nucleic acid is amplified by PCR and the amplified nucleic acid is detected subsequently (*e.g.,* detection occurs after target amplification).

PCR-flap assays include the QuARTS assays described in U.S. Pat. Nos. 8,361,720; 8,715,937; 8,916,344, and 9,212,392, and the amplification assays of US Pat. No. 9,096,893 (for example, as diagrammed in Figure 1 of that patent.) LQAS assays are flap assays using probe oligonucleotides having a longer target-specific region (Long probe Quantitative Amplified Signal, "LQAS") and TELQAS assays combine the LQAS probe oligos with a pre-amplification step (Target Enrichment Long probe Quantitative Amplified Signal), as is described in U.S. Pat. No. 10,648,025, and in WO 2020/112869, each of which is incorporated herein by reference in its entirety. In some embodiments, the PCR-flap assays are performed in a similar manner to the QuARTS assay and LQAS assays referenced above, with the exception that a FRET cassette or a FRET reporter is not used to detect cleavage (*e.g.,* not used to detect the cleaved flap sequence) but rather a ligatable molecule, such as a circularizable molecule, a padlock probe, a dumbbell probe, a linear vector, or one or a pair of oligonucleotides of a vector pair, is used to detect cleavage. Exemplary conditions for analysis of DNA and RNA target nucleic acids using PCR-flap assays are provided, for example, in U.S. Pat.10,648,025, and WO 2021/041726. Each of the above-named patents and patent publications is incorporated herein by reference in its entirety for all purposes.

As used herein, the term "PCR-flap assay reagents" refers to one or more reagents for detecting target sequences in a PCR-flap assay, the reagents comprising nucleic acid molecules capable of participating in amplification of a target nucleic acid and in formation of a flap cleavage structure in the presence of the target sequence, in a mixture containing DNA polymerase, a flap endonuclease (*e.g.,* a FEN-1 endonuclease), and a ligatable molecule (*e.g.,* a circularizable molecule, a padlock probe, a dumbbell probe, a linear vector, or one or a pair of oligonucleotides of a vector pair). In some embodiments, the reagents further comprise a bridge oligonucleotide. In some embodiments, the reagents further comprise a ligase.

As used herein, the term "padlock probe" refers to any oligonucleotide probe that can be used in formation of a nicked circle by hybridization to a strand of nucleic acid, and which by treatment that includes a ligase forms a covalently closed circular molecule. See, *e.g.,* M. Nilsson, *et al.* "Padlock probes: circularizing oligonucleotides for localized DNA detection." *Science*. 265 (5181): 2085–2088 (1994). In some embodiments, a padlock probe requires modification prior to being ligatable, *e.g.,* by endo- or exonuclease digestion (*e.g.,* to remove a flap sequence), polymerase extension (*e.g.,* to fill gaps) or other treatment to produce a circular probe comprising a ligatable nick. Preferably, the modification to produce the ligatable nick is dependent on the padlock probe being hybridized to an intended target nucleic acid. Padlock probes are described in U.S. Pat. No. 5,854,033 (Lizardi), WO99/49079 (Landegren) and U.S. Pat. No. 5,871,921 (Landegren & Kwiatkowski). A version of a padlock probe known as an inversion probe is described in U.S. Pat. No. 6,858,412 (Willis *et al*.). Inversion probes are padlock probes containing a cleavage site in the probe backbone, allowing the circularized probe to be cleaved to form a linear product, which may then be amplified and detected. In some embodiments, a padlock probe is used for detection of the amplified target by a PCR-flap assay, wherein the padlock probe comprises a 5′ end that is ligated to a first end (*e.g.,* a 5′ end or a 3′ end) of a cleaved flap sequence and a 3′ end that is ligated to a second end of the cleaved flap sequence, thereby forming a closed circular structure. In some embodiments, formation of the closed circular structure is assisted by use of a gap filling oligonucleotide, *e.g.,* a cleaved flap sequence, which is hybridized along with the padlock probe to a strand of nucleic acid that positions the ends of the gap-filling oligonucleotide and the padlock probe to form ligatable

nicks at each end of the gap-filling oligonucleotide. Upon ligation, the circular nucleic acid comprises the padlock probe and the gap-filling sequence.

In some embodiments a padlock probe and a gap-filling oligonucleotide hybridize together to a target nucleic acid, and in some embodiments, the padlock probe and gap-filling oligonucleotide hybridize together to a non-target oligonucleotide referred to as a "bridge" or "splint" oligonucleotide. See, *e.g.*, Kuhn, *et al.*, Nucleic Acids Research, 2002, Vol. 30, No. 2:574 (2002); Yixiao Cui, *et al*., "Cyclization of secondarily structured oligonucleotides to single-stranded rings by using *Taq* DNA ligase at high temperatures," RSC. Adv. 8:18972-79 (2018); I. Tabuchi, *et al*., "An Efficient Ligation Method in the Making of an *in vitro* Virus for *in vitro* Protein Evolution" *Biol. Proced. Online* ;4(1): 49-54. (2002); M. Szemes, *et al*., Diagnostic application of padlock probes - multiplex detection of plant pathogens using universal microarrays" *Nucleic Acids Research* 33(8): e70 (2005), and Lohman, et al., US Pat. No. 10,597,650, each of which is incorporated herein by reference it its entirety, for all purposes. In some embodiments, the closed circular structure is amplified and/or detected, for instance, by PCR, RCA, RCT, sequencing, *etc.*

Bridge oligonucleotides may comprise DNA or RNA nucleotides or analogs thereof, *e.g.*, 2′-O-methyl nucleotides, 2′-O-methoxyethyl nucleotides, phosphorothioate nucleotides, or nucleotides comprising base analogs, or combinations thereof, *e.g.*, strands of natural DNA or RNA bases comprising one or more positions or regions of 2′-O-methyl nucleotides. In some embodiments, nucleotides used in splint oligonucleotides may be selected to provide an A-form helical form or a B-form helical form, or combinations thereof, *e.g.*, when hybridized to complementary nucleic acid strands. For example, hybridization of RNA or RNA analog strands to DNA generally provides an A-form structure, while hybridization of DNA or DNA-analog strands to DNA generally provides a B-form structure, and chimerical strands having, for example, a region of RNA and a region of DNA may, when hybridized to a strand of DNA, provide a duplex having a region in A-form that transitions into a region in B-form. See, *e.g.*, Shaw, et al., "Recognition of the unique structure of DNA:RNA hybrids," *Biochimie* 90:1026-1039 (2008), which is incorporated herein by reference in its entirety, for all purposes.

As used herein in reference to cleavage of an invasive cleavage structure, the term "target cleavage site" refers to a preferred site (or sites) of cleavage on a nucleic acid structure (*e.g.,* an invasive cleavage structure) by a structure-specific nuclease (*e.g.,* a FEN-1 endonuclease) that recognizes the structure as a cleavage substrate. For example, as discussed by Kaiser, *et al*., 5′ flap endonucleases, including FEN-1 endonucleases, typically cleave an invasive cleavage structure in the downstream nucleic acid, generally after the first base-paired nucleotide, *i.e.,* one nucleotide into the downstream duplex. The target cleavage site is selected such that cleavage at the site releases the cleaved flap sequence, thereby making the cleaved flap sequence available for subsequent detection. For example, in some embodiments the flap sequence is positioned within the flap oligonucleotide such that upon cleavage of the invasive cleavage structure at a target cleavage site, the flap oligonucleotide is cleaved to produce (release) a cleaved flap from the target-specific portion of the oligonucleotide. Thus, the cleaved flap sequence is available for hybridization.

In some embodiments, the cleavage site in the invasive cleavage structure is such that cleavage of the flap sequence results in formation of a cleaved flap sequence that contains one additional base compared to the uncleaved flap sequence. For example, in some embodiments the cleavage site is such that cleavage of the flap sequence adds the 5′ terminal base of the target-specific region to the cleaved flap sequence. Accordingly, in some embodiments the flap sequence (when uncleaved from the flap oligonucleotide) comprises 14 bases, and cleavage by an endonuclease results in formation of a cleaved flap sequence comprising 15 bases. In preferred embodiments, an invasive cleavage structure is cleavable at a target cleavage site by a FEN-1 endonuclease from *Archaeoglobus fulgidus* ("*Afu* FEN-1") in a Mg^{++} flap assay buffer, as discussed below. See also, *e.g.,* U.S. Pat. No. 6,562,611 to Kaiser, *et al*., and Kaiser M.W., *et al*. (1999) J. Biol. Chem., 274:21387, each of which is incorporated herein by reference in its entirety for all purposes.

As used herein, "first paired nucleotide" and "first paired base," as used in reference to a FEN-1 cleavage site of an invasive cleavage structure, *e.g.,* in a padlock probe or flap oligonucleotide, refers to 5′-most base-paired nucleotide of the probe or oligonucleotide when hybridized in the downstream duplex of the cleavage structure, as illustrated, *e.g.,* in Figs. 1 and 2.

Detection of cleaved flaps is not limited to any particular methods. In some embodiments, for example, cleaved flaps may be used to form additional cleavage structures, *e.g.,* with a reporter molecule such as a FRET cassette, or may be used in formation of a promoter to enable transcription as a means of reporting signal. See, *e.g.,* US Pat. No. 6,872,816, which is incorporated herein by reference for all purposes. In some embodiments, the cleaved flaps serve as a primers for subsequent amplification. In some embodiments, the cleaved flaps are specifically hybridized to circular molecules to prime, *e.g.,* rolling circle replication as a means of creating a signal representing the presence of a target nucleic acid in a sample. In some embodiments, the cleaved flap hybridizes to a bridge oligonucleotide or bridge sequence to form ligatable nicks with the two ends of padlock or dumbbell probes, the nicks being subsequently ligated. For example, the cleaved flap sequences can be ligated to matched padlock probes or to dumbbell probes, thereby forming a closed circular structure for each flap sequence, each of which can then be amplified for subsequent detection of the target-specific cleavage events. In some embodiments, the cleaved flaps hybridize to a bridge oligonucleotide, *e.g.*, to a DNA oligonucleotide, an RNA oligonucleotide, or to a chimeric molecule comprising an RNA portion and a DNA portion, such that they can be ligated to a vector pair of oligonucleotides to form a linear ligated product. See, *e.g.*, FIG. 14.

As used herein, "methylation" refers to cytosine methylation at positions C5 or N4 of cytosine, the N6 position of adenine, or other types of nucleic acid methylation. In vitro amplified DNA is usually unmethylated because typical in vitro DNA amplification methods do not retain the methylation pattern of the amplification template. However, "unmethylated DNA" or "methylated DNA" can also refer to amplified DNA whose original template was unmethylated or methylated, respectively.

Accordingly, as used herein a "methylated nucleotide" or a "methylated nucleotide base" refers to the presence of a methyl moiety on a nucleotide base, where the methyl moiety is not present in a recognized typical nucleotide base. For example, cytosine does not contain a methyl moiety on its pyrimidine ring, but 5-methylcytosine contains a methyl moiety at position 5 of its pyrimidine ring. Therefore, cytosine is not a methylated nucleotide and 5-methylcytosine is a methylated nucleotide. In another example, thymine contains a methyl moiety at position 5 of its

pyrimidine ring; however, for purposes herein, thymine is not considered a methylated nucleotide when present in DNA since thymine is a typical nucleotide base of DNA.

As used herein, a "methylated nucleic acid molecule" refers to a nucleic acid molecule that contains one or more methylated nucleotides.

As used herein, a "methylation state," "methylation profile," and "methylation status" of a nucleic acid molecule refers to the presence or absence of one or more methylated nucleotide bases in the nucleic acid molecule. For example, a nucleic acid molecule containing a methylated cytosine is considered methylated (*e.g.,* the methylation state of the nucleic acid molecule is methylated). A nucleic acid molecule that does not contain any methylated nucleotides is considered unmethylated. In some embodiments, a nucleic acid may be characterized as "unmethylated" if it is not methylated at a specific locus (*e.g.,* the locus of a specific single CpG dinucleotide) or specific combination of loci, even if it is methylated at other loci in the same gene or molecule.

The methylation state of a particular nucleic acid sequence (*e.g.,* a gene marker or DNA region as described herein) can indicate the methylation state of every base in the sequence or can indicate the methylation state of a subset of the bases (*e.g.,* of one or more cytosines) within the sequence, or can indicate information regarding regional methylation density within the sequence with or without providing precise information of the locations within the sequence the methylation occurs. As used herein, the terms "marker gene" and "marker" are used interchangeably to refer to DNA, RNA, or protein (or other sample components) that is associated with a condition, *e.g.,* cancer or other disease, regardless of whether the marker region is in a coding region of DNA. Markers may include, *e.g.,* regulatory regions, flanking regions, intergenic regions, *etc.* Similarly, the term "marker" used in reference to any component of a sample, *e.g.,* protein, RNA, carbohydrate, small molecule, *etc.,* refers to a component that can be assayed in a sample (*e.g.,* measured or otherwise characterized) and that is associated with a condition of a subject, or of the sample from a subject. The term "methylation marker" refers to a gene or DNA in which the methylation state of the gene or DNA is associated with a condition, *e.g.,* cancer or other disease.

The methylation state of a nucleotide locus in a nucleic acid molecule refers to the presence or absence of a methylated nucleotide at a particular locus in the nucleic acid molecule. For example, the methylation state of a cytosine at the 7th nucleotide in a nucleic acid molecule is methylated when the nucleotide present at the 7th nucleotide in the nucleic acid molecule is 5-methylcytosine. Similarly, the methylation state of a cytosine at the 7th nucleotide in a nucleic acid molecule is unmethylated when the nucleotide present at the 7th nucleotide in the nucleic acid molecule is cytosine (and not 5-methylcytosine).

The methylation status can optionally be represented or indicated by a "methylation value" (*e.g.,* representing a methylation frequency, fraction, ratio, percent, *etc.*) A methylation value can be generated, for example, by quantifying the amount of intact nucleic acid present following restriction digestion with a methylation dependent restriction enzyme or by comparing amplification profiles after bisulfite reaction or by comparing sequences of bisulfite-treated and untreated nucleic acids. Accordingly, a value, *e.g.,* a methylation value, represents the methylation status and can thus be used as a quantitative indicator of methylation status across multiple copies of a locus. This is of particular use when it is desirable to compare the methylation status of a sequence in a sample to a threshold or reference value.

As used herein, "methylation frequency" or "methylation percent (%)" refers to the number of instances in which a molecule or locus is methylated relative to the number of instances the molecule or locus is unmethylated.

As such, the methylation state describes the state of methylation of a nucleic acid (*e.g.,* a genomic sequence). In addition, the methylation state refers to the characteristics of a nucleic acid segment at a particular genomic locus relevant to methylation. Such characteristics include, but are not limited to, whether any of the cytosine (C) residues within this DNA sequence are methylated, the location of methylated C residue(s), the frequency or percentage of methylated C throughout any particular region of a nucleic acid, and allelic differences in methylation due to, *e.g.,* difference in the origin of the alleles. The terms "methylation state," "methylation profile," and "methylation status" also refer to the relative concentration, absolute concentration, or pattern of methylated C or unmethylated C throughout any particular region of a nucleic acid in a biological sample. For example, if the cytosine (C) residue(s) within a nucleic acid sequence are

methylated it may be referred to as "hypermethylated" or having "increased methylation," whereas if the cytosine (C) residue(s) within a DNA sequence are not methylated it may be referred to as "unmethylated," "hypomethylated" or having "decreased methylation." Likewise, if the cytosine (C) residue(s) within a nucleic acid sequence are methylated as compared to another nucleic acid sequence (*e.g.,* from a different region or from a different individual, *etc.*) that sequence is considered hypermethylated or having increased methylation compared to the other nucleic acid sequence. Alternatively, if the cytosine (C) residue(s) within a DNA sequence are not methylated as compared to another nucleic acid sequence (*e.g.,* from a different region or from a different individual, *etc.*) that sequence is considered hypomethylated or having decreased methylation compared to the other nucleic acid sequence. Additionally, the term "methylation pattern" as used herein refers to the collective sites of methylated and unmethylated nucleotides over a region of a nucleic acid. Two nucleic acids may have the same or similar methylation frequency or methylation percent but have different methylation patterns when the number of methylated and unmethylated nucleotides is the same or similar throughout the region but the locations of methylated and unmethylated nucleotides are different. Sequences are said to be "differentially methylated" or as having a "difference in methylation" or having a "different methylation state" when they differ in the extent (*e.g.,* one has increased or decreased methylation relative to the other), frequency, or pattern of methylation. The term "differential methylation" refers to a difference in the level or pattern of nucleic acid methylation in a cancer or other disease positive sample as compared with the level or pattern of nucleic acid methylation in a cancer or other disease negative sample. It may also refer to the difference in levels or patterns between patients that have recurrence of cancer or other disease after surgery or other treatment versus patients who do not have recurrence. Differential methylation and specific levels or patterns of DNA methylation are prognostic and predictive biomarkers, *e.g.,* once the correct cut-off or predictive characteristics have been defined.

Methylation state frequency can be used to describe a population of individuals or a sample from a single individual. For example, a nucleotide locus having a methylation state frequency of 50% is methylated in 50% of instances and unmethylated in 50% of instances. Such a frequency can be used, for example, to describe the degree to which a nucleotide locus or nucleic acid region is methylated in a population of individuals or a collection of nucleic acids.

Thus, when methylation in a first population or pool of nucleic acid molecules is different from methylation in a second population or pool of nucleic acid molecules, the methylation state frequency of the first population or pool will be different from the methylation state frequency of the second population or pool. Such a frequency also can be used, for example, to describe the degree to which a nucleotide locus or nucleic acid region is methylated in a single individual. For example, such a frequency can be used to describe the degree to which a group of cells from a tissue sample are methylated or unmethylated at a nucleotide locus or nucleic acid region.

As used herein a "nucleotide locus" refers to the location of a nucleotide in a nucleic acid molecule. A nucleotide locus of a methylated nucleotide refers to the location of a methylated nucleotide in a nucleic acid molecule.

Typically, methylation of human DNA occurs on a dinucleotide sequence including an adjacent guanine and cytosine where the cytosine is located 5′ of the guanine (also termed CpG dinucleotide sequences). Most cytosines within the CpG dinucleotides are methylated in the human genome, however some remain unmethylated in specific CpG dinucleotide rich genomic regions, known as CpG islands (see, *e.g.,* Antequera, *et al*. (1990) *Cell* 62: 503–514).

As used herein, a "CpG island" refers to a G:C-rich region of genomic DNA containing an increased number of CpG dinucleotides relative to total genomic DNA. A CpG island can be at least 100, 200, or more base pairs in length, where the G:C content of the region is at least 50% and the ratio of observed CpG frequency over expected frequency is 0.6; in some instances, a CpG island can be at least 500 base pairs in length, where the G:C content of the region is at least 55% and the ratio of observed CpG frequency over expected frequency is 0.65. The observed CpG frequency over expected frequency can be calculated according to the method provided in Gardiner-Garden *et al* (1987) *J. Mol. Biol.* 196: 261–281. For example, the observed CpG frequency over expected frequency can be calculated according to the formula $R = (A \times B) / (C \times D)$, where R is the ratio of observed CpG frequency over expected frequency, A is the number of CpG dinucleotides in an analyzed sequence, B is the total number of nucleotides in the analyzed sequence, C is the total number of C nucleotides in the analyzed sequence, and D is the total number of G nucleotides in the analyzed sequence. Methylation state is typically determined in CpG islands, *e.g.,* at promoter regions. It will be appreciated though that other

sequences in the human genome are prone to DNA methylation such as CpA and CpT (see Ramsahoye (2000) *Proc. Natl. Acad. Sci. USA* 97: 5237–5242; Salmon and Kaye (1970) *Biochim. Biophys. Acta.* 204: 340–351; Grafstrom (1985) *Nucleic Acids Res.* 13: 2827–2842; Nyce (1986) *Nucleic Acids Res.* 14: 4353–4367; Woodcock (1987) *Biochem. Biophys. Res. Commun.* 145: 888-894).

As used herein, the terms "methyl cytosine," "methyl C," "methylated cytosine," "methylated C," and "meC" are used interchangeably and encompass both 5-methylcytosine (5mC) and 5-hydroxymethyl cytosine (5hmC).

As used herein, the term "modified cytosine" or "modified C" refers to a cytosine nucleobase in which the base portion has a side group or other modification as compared to a standard cytosine nucleotide. Modified cytosines include but are not limited to 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), 5-carboxylcytosine (5caC), and 5-formylcytosine (5fC).

As used herein, a "methylation-specific reagent" refers to a reagent that modifies a nucleotide of the nucleic acid molecule as a function of the methylation state of the nucleic acid molecule. In particular embodiments the term refers to a compound or composition or other agent or collection or sequence thereof that can change the nucleotide sequence of a nucleic acid molecule in a manner that reflects the methylation state of the nucleic acid molecule. Methods of treating a nucleic acid molecule with such reagents can include contacting the nucleic acid molecule with the reagent, coupled with additional steps, if desired, to accomplish the desired change of nucleotide sequence. Such methods can be applied in a manner in which unmethylated nucleotides (*e.g.,* each unmethylated cytosine) is modified to a different nucleotide. For example, in some embodiments, such a reagent can deaminate unmethylated cytosine nucleotides to produce deoxyuracil residues. An exemplary reagent is a bisulfite reagent.

In addition, treatment with a "methylation-specific reagent" can be applied in a manner in which methylated nucleotides are modified to a different nucleotide. For example, methylated cytosines (including 5mC and 5hmC) in DNA can be converted by combining oxidation by ten-eleven translocation (TET) family dioxygenases with reduction by borane derivatives (*e.g.,* pyridine borane and 2-picoline borane (pic-BH_3)), in a process referred to herein as TAPS (**T**ET

Assisted **P**yridine borane **S**equencing). See, *e.g.,* the TAPS method combining oxidation by TET enzymes with reduction by borane derivatives, described, *e.g.,* in US 2020/0370114 A1, Application Ser. No. 16/960,510, filed July 7, 2020, which is incorporated herein by reference for all purposes. In embodiments of the TAPS method, methylated cytosines are converted to dihydrouracil. Other methods of converting methylated Cs include, for example:

Sequencing Method	**Cytosine Modification**	**Method of Analysis**
TET-assisted bisulfite sequencing (TAB-seq)	5hmC	Enzymatic treatment with T4-BGT then TET followed by bisulfite treatment
Oxidative bisulfite sequencing (oxBS)	5mC	Treatment with an oxidation reagent followed by bisulfite treatment
APOBEC-coupled epigenetic sequencing (ACE-seq)	5hmC	Enzymatic treatment with T4-BGT and APOBEC3A

(Loise Williams, *et al.*, Enzymatic Methyl-seq: The next generation of methylome analysis, New England Biolabs Expressions 2019. Feature Article.)

In preferred embodiments, methylation-specific reagents modify one nucleotide of the four typically-occurring nucleotides in a nucleic acid molecule (C, G, T, and A for DNA and C, G, U, and A for RNA), such that the reagent modifies the one nucleotide without modifying the other three nucleotides. The nucleotides resulting from conversion are not limited to the four typically occurring nucleotides listed above, and may include, for example, modified or variant forms of purine or pyrimidine structures, including, *e.g.,* nucleobase analogs discussed herein. In preferred embodiments, the nucleotides produced by conversion are recognized by DNA modifying enzymes, *e.g.,* DNA polymerases, as one of the typically occurring nucleotides listed above, and can serve as templates for strand replication. Conversion of nucleotides by any of the methods described herein may be detected by determining the sequence of a resulting strand, *e.g.,* using standard sequencing methods, or by interrogating single or a few specific nucleotide locations to determine the identity of the nucleobase at the select locations.

As used herein, the term "converted" as used in reference to a nucleotide or DNA strand refers to a nucleotide or DNA strand that has been treated with a reagent or reagents under conditions in which some nucleotides are converted into other nucleotides. For example, in bisulfite conversion, cytosine bases in the DNA are typically deaminated, resulting in uracil bases at converted loci. While inefficient, bisulfite can also cause deamination of 5-methyl cytosine bases, resulting in thymine bases at converted loci. "Bisulfite-treated" and bisulfite-converted" are used interchangeably herein in reference to DNA or nucleotide loci that have been exposed to a bisulfite reagent under conditions in which cytosine is typically converted to uracil. In the bisulfite-free TAPS process, methylated cytosines are selectively converted to dihydrouracil (DHU), while unmethylated Cs are not converted. The DHU nucleotides base pair with A nucleotides rather than G nucleotides, making them readily distinguishable from the unmethylated C bases in the converted DNA strands.

As used herein, the term "poorly converted," as used in reference to conversion of a nucleotide upon treatment with reagent(s) and/or conditions under which some nucleotides are converted into other nucleotides, refers to a nucleotide having a reduced rate of conversion (preferably less than 10%, more preferably less than 1% of the rate of conversion) under the given treatment, as compared to the rate of conversion of a nucleotide expected to convert under the same treatment. For example, bisulfite-mediated deamination of cytosine is greatly slowed down by the presence of a 5-methyl group, such that the rate for the deamination of 5-methylcytosine to form thymine is about two orders of magnitude smaller than the rate for deamination of cytosine to form uracil (see, *e.g.,* Hayatsu *et al*., Biochemistry 18:4:632-37 (1979); Hayatsu, Proc. Jpn. Acad 84(8):321-330 (2008), each of which is incorporated herein by reference). Thus, 5-methylcytosine is said to be poorly converted compared to cytosine under bisulfite treatment conditions typically used to convert cytosine to uracil.

The term "bisulfite reagent" refers to a reagent comprising bisulfite, disulfite, hydrogen sulfite, or combinations thereof, useful as disclosed herein to distinguish between methylated and unmethylated CpG dinucleotide sequences. Methods of said treatment are known in the art (*e.g.,* PCT/EP2004/011715 and WO 2013/116375, each of which is incorporated by reference in its entirety). In some embodiments, bisulfite treatment is conducted in the presence of denaturing solvents such as but not limited to n-alkyleneglycol or diethylene glycol dimethyl ether (DME),

or in the presence of dioxane or dioxane derivatives. In some embodiments the denaturing solvents are used in concentrations between 1% and 35% (v/v). In some embodiments, the bisulfite reaction is carried out in the presence of scavengers such as but not limited to chromane derivatives, *e.g.,* 6-hydroxy-2,5,7,8,-tetramethylchromane 2-carboxylic acid or trihydroxybenzone acid and derivatives thereof, *e.g.,* Gallic acid (see: PCT/EP2004/011715, which is incorporated by reference in its entirety). In certain preferred embodiments, the bisulfite reaction comprises treatment with ammonium hydrogen sulfite, also referred to as ammonium bisulfite, *e.g.,* as described in WO 2013/116375.

The term "methylation assay" refers to any assay for determining the methylation state of one or more CpG dinucleotide sequences within a sequence of a nucleic acid.

The terms "target" and "template" as used in reference to a nucleic acid strand of a flap structure, *e.g.,* an invasive cleavage structure, are used interchangeably, and refer to the strand of nucleic acid to which upstream and downstream nucleic acids or nucleic acid regions hybridize to form an invasive cleavage structure. A target nucleic acid may be isolated from a sample, *e.g.*, a patient sample, or the target nucleic acid may be a copied or amplified nucleic acid strand. For example, in a PCR-flap assay, replicated DNA strands produced in an amplification reaction mixture are also target nucleic acids on which an invasive cleavage structure is formed by hybridization of a flap oligonucleotide to the target strand adjacent to an upstream nucleic acid (*e.g.*, an upstream invasive oligonucleotide or a primer that is extended by a polymerase to become an invasive oligonucleotide). While a template strand may serve as template for extension of a primer by a polymerase, *e.g.,* in a PCR-flap endonuclease assay, use of the term "template" is not limited to polymerizing assays or reactions. When used in reference to ligation, a template refers to a strand of nucleic acid to which 3′ and 5′ ends of complementary strands are hybridized, forming a ligatable nick.

As used herein, the term "3′ end blocker" refers to a 3′ end modification, *e.g.,* on an oligonucleotide, that suppresses cleavage by a flap endonuclease of an invasive cleavage structure when the 3′ end blocker is present on the 3′ end of an upstream nucleic acid in the cleavage structure. For example, 3′ amines, 3′ phosphate (3′-PO_4), 3′ biotin, 3′ C_6 (a 6-carbon

glycol spacer, also referred to as "3′ hexanediol"), and 3′ dideoxynucleotides suppress flap cleavage if present on an upstream nucleic acid. See, *e.g.,* Kaiser, *et al*., *supra*.

As used herein, the term "5′ blocking group" refers to a 5′ end modification or moiety that prevents a phosphate at a 5′ end of a nucleic acid strand or at the 5′ end of a region of a nucleic acid strand (*e.g.*, the 5′ end of a duplexed portion of a padlock probe in a downstream duplex, when the 5′ end of the padlock probe includes an unpaired 5′ flap sequence), from being available for enzymatic ligation, *e.g.*, to an hydroxyl at the 3′ end of a nucleic acid strand, *e.g.*, the 3′ terminal nucleotide of an adjacent duplexed portion of a padlock probe. Exemplary 5′ blocking groups include but are not limited to a carbon chain (*e.g.*, hexane), a non-standard nucleotide, an abasic nucleotide (*e.g.*, a ribose or deoxyribose lacking a nucleobase), a dye tag, a flap nucleic acid sequence, a hairpin flap, *etc.* In preferred embodiments, a 5′ blocking group is attached to the 5′ position of a nucleotide (*e.g.*, the 5′ end of a duplexed portion of a padlock probe in a downstream duplex) via a phosphate, *e.g.*, a phosphodiester bond. In particularly preferred embodiments the 5′ blocking group is attached to the 5′ position of a nucleotide that is 5′ of a FEN-1 cleavage site in an invasive cleavage structure.

As used herein, the term "Mg^{++} flap assay buffer" refers to a buffer solution for a flap endonuclease that includes Mg^{++} as the predominant or essentially only divalent cation in the buffer, such that assay reactions performed in the buffer, *e.g.,* flap assays, exhibit characteristics of the presence of Mg^{++} (*e.g.,* high specificity of some flap endonucleases for invasive cleavage structures as compared to, *e.g.,* Y-structures, pseudo Y structures), and do not exhibit characteristics associated with the presence of other divalent cations (*e.g.,* alternative cleavage activities of flap endonucleases associated with use of Mn^{++} with or in place of Mg^{++}), as described, *e.g.,* by Kaiser, *et al*., *supra*. In some embodiments the flap assay buffer has very low or no KCl and comprises elevated Mg^{++} as compared to standard PCR buffer, (*e.g.,* a flap assay buffer of the technology comprises >6 mM, preferably > 7mM, more preferably 7.5mM Mg^{++}, while PCR buffers typically comprise about 1.5 to 2.5 mM Mg^{++}).

The term "low-bias amplification buffer" as used herein refers to an amplification buffer composed to exhibit low target-to-target variation in amplification efficiency for different targets amplified together in a multiplexed amplification reaction, *e.g.*, a multiplexed preamplification

reaction prior to a follow-on amplification reaction, *e.g.*, a PCR-flap assay reaction. Amplification bias between different targets can be assessed by measuring the efficiencies of amplification for different targets of known concentration. In some embodiments of the present technology, low-bias amplification buffer is a buffer useful for PCR-flap assays and comprising a high magnesium concentration (*e.g.*, at least 6 mM, preferably 6 to 10 mM, preferably, 7 to 9 mM, preferably at about 7.5 mM as a final concentration in a reaction mixture), in contrast to PCR buffers that typically comprise about 1 to 4 mM final concentration of magnesium in a reaction mixture. In preferred embodiments, a low-bias amplification buffer comprises 3-(n-morpholino) propanesulfonic acid (MOPS) buffer, and in certain preferred embodiments, a low-bias amplification buffer comprises 7.5 mM $MgCl_2$, 10 mM MOPS, 0.3 mM Tris-HCl, pH 8.0, 0.8 mM KCl, 0.1 μg/μL BSA, 0.0001% Tween-20, and 0. 0001% IGEPAL CA-630. Herein the terms "PCR-flap assay buffer" and "low-bias amplification buffer" are used interchangeably.

Ranges for the amount of Mg^{++} used in a low-bias amplification buffer include any concentration encompassed by the ranges discussed above. For example, a low-bias amplification buffer may contain 6, 6.1. 6.2, 6.5, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9.0, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10.0, 10.1, 10.2, 10.3, 10.4, 10.5, 10.6, 10.7, 10.8, 10.9, 11.0 mM Mg^{++}, or more, or any fractional value concentration therebetween. These concentrations are recited by way of example and not of limitation.

As used herein, "low-bias amplification buffer" is distinct from PCR buffers that comprise Mg^{++} above about 4 mM, but that further comprise reagents that reduce the helix-stabilizing effect of a high Mg^{++} concentration, *e.g.*, DMSO, $(NH_4)_2SO_4$, betaine, *etc.*, sometimes referred to as enhancer reagents. For discussion of such enhancer reagents, see, *e.g.*, Ralser, *et al*., An efficient and economic enhancer mix for PCR, Biochemical and Biophysical Research Communications 347 (2006) 747–751; O. Henegariu, *et al*., "Multiplex PCR: Critical Parameters and Step-by-Step Protocol," *BioTechniques 23:504-511 (September 1997);* and Qiagen PCR Brochure "Maximizing PCR and RT-PCR Success," Third Edition (Document 1104683, 10/2016), each of which is incorporated herein by reference in its entirety. In preferred embodiments, low-bias amplification buffer is essentially free of $(NH_4)_2SO_4$. In some

embodiments, a low-bias amplification buffer is essentially free of ammonium ions. In some embodiments, low-bias amplification buffer is essentially free of DMSO and/or betaine.

As used herein, the term "essentially free of" as used in reference to a component excluded from a composition, *e.g.*, a reaction mixture, a buffer, *etc.*, refers to a composition that is not formulated to include the excluded component, but that may comprise trace amounts of the component, *e.g.*, by carry-over from an earlier reaction step, or from a concentrated stock of a reaction component that is added in a small amount, *e.g.*, an enzyme in a storage solution. For example, a small amount of glycerol, or other material may be introduced into a reaction mixture (*e.g.*, a PCR flap assay reaction mixture) by addition of an aliquot of an enzyme (*e.g.*, a polymerase) that includes the material for stable storage, but such materials are typically diluted sufficiently in the reaction mixture that the trace amounts of glycerol and other material do not alter the expected function of the reaction mixture, *e.g.*, a trace amount of $(NH_4)_2SO_4$, betaine, or DMSO, would not be expected to alter the low-bias multiplex amplification results observed when a low-bias amplification buffer described herein is used. Such trace amounts of materials are typically not reported in literature as part of the formulation of the final reaction mixture. Thus, a composition that is "essentially free" of a recited component may contain the excluded component at a level such that, though present, it does not alter the function of the recited composition as compared to a pure composition, *i.e.,* a composition that is completely free of the excluded component.

As used herein, the term "flap assay reagents" or "invasive cleavage assay reagents" refers to a collection of all reagents required for performing a flap assay or invasive cleavage assay on a substrate. As is known in the art, flap assays generally include oligonucleotides for forming an invasive cleavage structure and a flap endonuclease. Flap assay reagents may optionally contain a target or template nucleic acid to which a flap oligonucleotide and an invasive oligonucleotide (or a flap oligonucleotide having an invasive oligonucleotide region) bind. In particularly preferred embodiments, flap assay reagents comprise a Mg^{++} flap assay buffer, as discussed herein. In some embodiments, flap assay reagents further comprise a ligatable molecule (*e.g.,* a circularizable molecule, a padlock probe, a dumbbell probe, a linear vector, one or a pair of oligonucleotides of a vector pair) that hybridizes to a cleaved flap sequence. In some embodiments, flap assay reagents further comprise a bridge oligonucleotide.

In some embodiments, the bridge oligonucleotide comprises a sequence that is complementary to the cleaved flap sequence. For example, in some embodiments the bridge oligonucleotide comprises an inner segment that is complementary to the cleaved flap sequence. In some embodiments, the bridge oligonucleotide comprises additional nucleotides 3′ and 5′ of the sequence complementary to the cleaved flap sequence (*e.g.,* 3′ and 5′ of the inner segment) that are complementary to the first target region and the second target region of ligatable molecule(s), (*e.g.,* a circularizable molecule, a padlock probe, a dumbbell probe, a linear vector, one or a pair of oligonucleotides of a vector pair). In some embodiments, the flap assay reagents further comprise a ligase.

As used herein, the term "FRET" refers to fluorescence resonance energy transfer, a process in which moieties (*e.g.,* fluorophores) transfer energy *e.g.,* among themselves, or, from a fluorophore to a non-fluorophore (*e.g.,* a quencher molecule). In some circumstances, FRET involves an excited donor fluorophore transferring energy to a lower-energy acceptor fluorophore via a short-range (*e.g.,* about 10 nm or less) dipole-dipole interaction. In other circumstances, FRET involves a loss of fluorescence energy from a donor and an increase in fluorescence in an acceptor fluorophore. In still other forms of FRET, energy can be exchanged from an excited donor fluorophore to a non-fluorescing molecule (*e.g.,* a quenching molecule). FRET is known to those of skill in the art and has been described (See, *e.g.,* Stryer *et al*., 1978, Ann. Rev. Biochem., 47:819; Selvin, 1995, Methods Enzymol., 246:300; Orpana, 2004 Biomol Eng 21, 45-50; Olivier, 2005 Mutant Res 573, 103-110, each of which is incorporated herein by reference in its entirety).

As used herein, the term "FRET system" refers to a pair or group of moieties that together act as donor-acceptor or donor-quencher partners for FRET-based analysis of molecules, *e.g.,* flap oligonucleotides or assay reporter molecules. The moieties of a FRET system finding application in the technology are not limited to these placements and may, for example, be switched in position, may be positioned on different parts of the illustrated molecules, or may comprise additional donor or acceptor moieties that are the same or different.

As used herein, the phrase "not substantially complementary" as used in reference to a probe flap or arm means that the flap portion is sufficiently non-complementary not to hybridize selectively to a nucleic acid sequence, *e.g.,* a target nucleic acid or amplified DNA, under the

designated annealing conditions or stringent conditions, encompassing the terms "substantially non-complementary" and "perfectly non-complementary."

The term "signal" as used herein refers to any detectable effect, such as would be caused or provided by a label or by action or accumulation of a component or product in an assay reaction.

The term "real time" as used herein in reference to detection of nucleic acid amplification or signal amplification refers to the detection or measurement of the accumulation of products or signal in the reaction while the reaction is in progress, *e.g.,* during incubation or thermal cycling. Such detection or measurement may occur continuously, or it may occur at a plurality of discrete points during the progress of the amplification reaction, or it may be a combination. For example, in a polymerase chain reaction, detection (*e.g.,* of fluorescence) may occur continuously during all or part of thermal cycling, or it may occur transiently, at one or more points during one or more cycles. In some embodiments, real time detection of PCR is accomplished by determining a level of fluorescence at the same point (*e.g.,* a time point in the cycle, or temperature step in the cycle) in each of a plurality of cycles, or in every cycle. Real time detection of amplification may also be referred to as detection "during" the amplification reaction.

As used herein, the term "abundance of nucleic acid" refers to the amount of a particular target nucleic acid sequence present in a sample or aliquot. The amount is generally referred to in terms of mass (*e.g.,* µg), mass per unit of volume (*e.g.,* µg/µL); copy number (*e.g.,* 1000 copies, 1 attomole), or copy number per unit of volume (*e.g.,* 1000 copies per mL, 1 attomole per µL). Abundance of a nucleic acid can also be expressed as an amount relative to the amount of a standard of known concentration or copy number. Measurement of abundance of a nucleic acid may be on any basis understood by those of skill in the art as being a suitable quantitative representation of nucleic acid abundance, including physical density or the sample, optical density, refractive property, staining properties, or on the basis of the intensity of a detectable label, *e.g.,* a fluorescent label.

The term "amplifying" or "amplification" in the context of nucleic acids refers to the production of multiple copies of a polynucleotide, or a portion of the polynucleotide, typically starting from a small amount of the polynucleotide (*e.g.,* a single polynucleotide molecule),

where the amplification products or amplicons are generally detectable. Amplification of polynucleotides encompasses a variety of chemical and enzymatic processes. The generation of multiple DNA copies from one or a few copies of a target or template DNA molecule during a polymerase chain reaction (PCR) or a ligase chain reaction (LCR; see, *e.g.,* U.S. Patent No. 5,494,810; herein incorporated by reference in its entirety) are forms of amplification. Additional types of amplification include, but are not limited to, allele-specific PCR (see, *e.g.,* U.S. Patent No. 5,639,611; herein incorporated by reference in its entirety), assembly PCR (see, *e.g.,* U.S. Patent No. 5,965,408; herein incorporated by reference in its entirety), helicase-dependent amplification (see, *e.g.,* U.S. Patent No. 7,662,594; herein incorporated by reference in its entirety), hot-start PCR (see, *e.g.,* U.S. Patent Nos. 5,773,258 and 5,338,671; each herein incorporated by reference in their entireties), intersequence-specific PCR, inverse PCR (see, *e.g.,* Triglia, *et al.*(1988) Nucleic Acids Res., 16:8186; herein incorporated by reference in its entirety), ligation-mediated PCR (see, *e.g.,* Guilfoyle, R. *et al*., Nucleic Acids Research, 25:1854-1858 (1997); U.S. Patent No. 5,508,169; each of which are herein incorporated by reference in their entireties), methylation-specific PCR (see, *e.g.,* Herman, *et al*., (1996) PNAS 93(13) 9821-9826; herein incorporated by reference in its entirety), miniprimer PCR, multiplex ligation-dependent probe amplification (see, *e.g.,* Schouten, *et al*., (2002) Nucleic Acids Research 30(12): e57; herein incorporated by reference in its entirety), multiplex PCR (see, *e.g.,* Chamberlain, *et al*., (1988) Nucleic Acids Research 16(23) 11141-11156; Ballabio, *et al*., (1990) Human Genetics 84(6) 571-573; Hayden, *et al*., (2008) BMC Genetics 9:80; each of which are herein incorporated by reference in their entireties), nested PCR, overlap-extension PCR (see, *e.g.,* Higuchi, *et al*., (1988) Nucleic Acids Research 16(15) 7351-7367; herein incorporated by reference in its entirety), real time PCR (see, *e.g.,* Higuchi, *et al.*, (1992) Biotechnology 10:413-417; Higuchi, *et al*., (1993) Biotechnology 11:1026-1030; each of which are herein incorporated by reference in their entireties), reverse transcription PCR (see, *e.g.,* Bustin, S.A. (2000) J. Molecular Endocrinology 25:169-193; herein incorporated by reference in its entirety), solid phase PCR, thermal asymmetric interlaced PCR, and Touchdown PCR (see, *e.g.,* Don, *et al*., Nucleic Acids Research (1991) 19(14) 4008; Roux, K. (1994) Biotechniques 16(5) 812-814; Hecker, *et al*., (1996) Biotechniques 20(3) 478-485; each of which are herein incorporated by reference in their entireties). Polynucleotide amplification also can be accomplished using digital

PCR (see, *e.g.,* Kalinina, *et al*., Nucleic Acids Research. 25; 1999-2004, (1997); Vogelstein and Kinzler, Proc Natl Acad Sci USA. 96; 9236-41, (1999); International Patent Publication No. WO05023091A2; US Patent Application Publication No. 20070202525; each of which are incorporated herein by reference in their entireties). In some embodiments, a portion of a target nucleic acid is copied in the amplification, and in some embodiments, a non-target polynucleotide is amplified in response to the presence of a target nucleic acid, (*e.g.,* a cleaved flap, ligation product, a rolling circle replication product, *etc.*)

The term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188, that describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic or other DNA or RNA, without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured, and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing, and polymerase extension can be repeated many times (*e.g.,* denaturation, annealing and extension constitute one "cycle;" there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" ("PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified" and are "PCR products" or "amplicons." Those of skill in the art will understand the term "PCR" encompasses many variants of the originally described method using, *e.g.,* real time PCR, nested PCR, reverse transcription PCR (RT-PCR), single primer and arbitrarily primed PCR, *etc.*

As used herein, the term "primer annealing" refers to conditions that permit oligonucleotide primers to hybridize to template nucleic acid strands, preferably sufficiently to be extended by a DNA polymerase. Conditions for primer annealing vary with the length and sequence of the primer and are generally based upon the T_m that is determined or calculated for the primer. For example, an annealing step in an amplification method that involves thermocycling involves reducing the temperature after a heat denaturation step to a temperature based on the T_m of the primer sequence, for a time sufficient to permit such annealing.

As used herein, the term "amplifiable nucleic acid" is used in reference to nucleic acids that may be amplified by any amplification method. It is contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

The term "amplicon" or "amplified product" refers to a segment of nucleic acid, generally DNA, generated by an amplification process such as the PCR process. The terms are also used in reference to RNA segments produced by amplification methods that employ RNA polymerases, such as NASBA, TMA, *etc.*

The term "amplification plot" as used in reference to a thermal cycling amplification reaction refers to the plot of signal that is indicative of amplification, *e.g.,* fluorescence signal, versus cycle number. When used in reference to a non-thermal cycling amplification method, an amplification plot generally refers to a plot of the accumulation of signal as a function of time.

The term "baseline" as used in reference to an amplification plot refers to the detected signal coming from assembled amplification reactions prior to incubation or, in the case of PCR, in the initial cycles, in which there is little change in signal.

The term "C_t" or "threshold cycle" as used herein in reference to real time detection during an amplification reaction that is thermal cycled refers to the fractional cycle number at which the detected signal (*e.g.,* fluorescence) passes the fixed threshold.

The term "no template control" and "no target control" (or "NTC") as used herein in reference to a control reaction refers to a reaction or sample that does not contain template or target nucleic acid. It is used to verify amplification quality.

As used herein, the term "sample template" refers to nucleic acid originating from a sample that is analyzed for the presence of "target." In contrast, "background template" is used in reference to nucleic acid other than sample template that may or may not be present in a

sample. The presence of background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

As used herein, the terms "patient" or "subject" refer to organisms to be subject to various tests provided by the technology. The term "subject" includes animals, preferably mammals, including humans. In a preferred embodiment, the subject is a primate. In an even more preferred embodiment, the subject is a human. Further with respect to diagnostic methods, a preferred subject is a vertebrate subject. A preferred vertebrate is warm-blooded; a preferred warm-blooded vertebrate is a mammal. A preferred mammal is most preferably a human. As used herein, the term "subject" includes both human and animal subjects. Thus, veterinary therapeutic uses are provided herein. As such, the present technology provides for the diagnosis of mammals such as humans, as well as those mammals of importance due to being endangered, such as Siberian tigers; of economic importance, such as animals raised on farms for consumption by humans; and/or animals of social importance to humans, such as animals kept as pets or in zoos. Examples of such animals include but are not limited to: carnivores such as cats and dogs; swine, including pigs, hogs, and wild boars; ruminants and/or ungulates such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels; pinnipeds; and horses. Thus, also provided is the diagnosis and treatment of livestock, including, but not limited to, domesticated swine, ruminants, ungulates, horses (including racehorses), and the like. The presently-disclosed subject matter further includes a system for diagnosing a condition, such as cancer or other disease, in a subject. The system can be provided, for example, as a commercial kit that can be used to screen for a risk of, or diagnose, a condition, such as cancer or other disease, in a subject from whom a biological sample has been collected. An exemplary system provided in accordance with the present technology includes assessing the methylation state of a marker described herein.

The term "sample" is used in its broadest sense. In one sense it can refer to an animal cell, tissue, or fluid. In another sense, it refers to a specimen or culture obtained from any source, as well as biological and environmental samples. Biological samples may be obtained from plants or animals (including humans) and encompass, *e.g.,* fluids, solids, tissues, and gases. Environmental samples include environmental material such as surface matter, soil, water, and

industrial samples. These examples are not to be construed as limiting the sample types applicable to the present invention. As used herein in reference to samples, the term "a sample" collected from a source or subject, *e.g.,* from a patient, is not limited to a single physical specimen but also encompasses a sample that is collected in multiple portions, *e.g.,* "a sample" of blood may be collected in two, three, four or more different blood collection tubes or other blood collection devices (*e.g.,* bags), or combinations of different blood collection devices. A sample "suspected of containing" a marker or other material may contain or not contain the marker.

The present technology is not limited by the type of biological sample used or analyzed. The present technology is useful with a variety of biological samples including, but not limited to, tissue (*e.g.,* organ (*e.g.,* heart, liver, brain, lung, stomach, intestine, spleen, kidney, pancreas, and reproductive organs), glandular, skin, and muscle), cell (*e.g.,* blood cell (*e.g.,* lymphocyte or erythrocyte), muscle cell, tumor cell, and skin cell), gas, bodily fluid (*e.g.,* blood or portion thereof, serum, plasma, urine, semen, saliva, *etc.*), or solid (*e.g.,* stool) samples obtained from a human (*e.g.,* adult, infant, or embryo) or animal (*e.g.,* cattle, poultry, mouse, rat, dog, pig, cat, horse, and the like). In some embodiments, biological samples may be solid food and/or feed products and/or ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may be obtained from all of the various families of domestic animals, as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, lagomorphs, rodents, pinnipeds, *etc.*

Biological samples also include biopsies and tissue sections (*e.g.,* biopsy or section of tumor, growth, rash, infection, or paraffin-embedded sections), medical or hospital samples (*e.g.,* including, but not limited to, blood samples, saliva, buccal swab, cerebrospinal fluid, pleural fluid, milk, colostrum, lymph, sputum, vomitus, bile, semen, oocytes, cervical cells, amniotic fluid, urine, stool, hair, and sweat), laboratory samples (*e.g.,* subcellular fractions), and forensic samples (*e.g.,* blood or tissue (*e.g.,* spatter or residue), hair and skin cells containing nucleic acids), and archeological samples (*e.g.,* fossilized organisms, tissue, or cells).

Environmental samples include, but are not limited to, environmental material such as surface matter, soil, water (*e.g.,* freshwater or seawater), algae, lichens, geological samples, air containing materials containing nucleic acids, crystals, and industrial samples, as well as samples

obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items.

Samples may be prepared by any desired or suitable method. In some embodiments, nucleic acids are analyzed directly from bodily fluids, stool, or other samples using the methods described in U.S. Pat. No. 9,000,146, which is herein incorporated by reference in its entirety for all purposes.

The above-described examples are not, however, to be construed as limiting the sample (*e.g.,* suspected of comprising a target sequence, gene, or template (*e.g.,* the presence or absence of which can be determined using the compositions and methods of the present technology)) types applicable to the present technology.

The term "nucleic acid" as used herein refers to any polynucleotide in which nucleosides are connected together via a sugar-phosphate backbone.

The terms "nucleic acid sequence" and "nucleic acid molecule" as used herein refer to an oligonucleotide, nucleotide or polynucleotide, and fragments or portions thereof. The terms encompass sequences that include analogs of DNA and RNA nucleotides, including those listed above, and also including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxyl-methyl)uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethyl-aminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudo-uracil, 1-methylguanine, 1-methylinosine, 2,2-dimethyl-guanine, 2-methyladenine, 2-methylguanine, 3-methyl-cytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxy-amino-methyl-2-thiouracil, beta-D-mannosylqueosine, 5′-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, 2,6-diaminopurine, and pyrazolo[3,4-d]pyrimidines such as guanine analogue 6 amino 1H-pyrazolo[3,4d]pyrimidin 4(5H) one (ppG or PPG, also Super G) and the adenine analogue 4 amino 1H-pyrazolo[3,4d]pyrimidine (ppA or PPA). The xanthine analogue 1H-pyrazolo[5,4d]pyrimidin 4(5H)-6(7H)-dione (ppX) can also be used. These base analogues,

when present in an oligonucleotide, strengthen hybridization, and improve mismatch discrimination. All tautomeric forms of naturally occurring bases, modified bases and base analogues may be included in the oligonucleotide conjugates of the technology. Other modified bases useful in the present technology include 6-amino-3-prop-1-ynyl-5-hydropyrazolo[3,4-d]pyrimidine-4-one, PPPG; 6-amino-3-(3-hydroxyprop-1-yny)l-5-hydropyrazolo[3,4-d]pyrimidine-4-one, HOPPPG; 6-amino-3-(3-aminoprop-1-ynyl)-5-hydropyrazolo[3,4-d]pyrimidine-4- -one, NH2PPPG; 4-amino-3-(prop-1-ynyl)pyrazolo[3,4-d]pyrimidine, PPPA; 4-amino-3-(3-hydroxyprop-1-ynyl)pyrazolo[3,4-d]pyrimidine, HOPPPA; 4-amino-3-(3-aminoprop-1-ynyl)pyrazolo[3,4-d]pyrimidine, NH_2 PPPA; 3-prop-1-ynylpyrazolo[3,4-d]pyrimidine-4,6-diamino, $(NH_2)_2$ PPPA; 2-(4,6-diaminopyrazolo[3,4-d]pyrimidin-3-yl)ethyn-1-ol, $(NH_2)_2$ PPPAOH; 3-(2-aminoethynyl)pyrazolo[3,4-d]pyrimidine-4,6-diamine, $(NH_2)_2$ $PPPANH_2$; 5-prop-1-ynyl-1,3-dihydropyrimidine-2,4-dione, PU; 5-(3-hydroxyprop-1-ynyl)-1,3-dihydropyrimidine-2,4-dione, HOPU; 6-amino-5-prop-1-ynyl-3-dihydropyrimidine-2-one, PC; 6-amino-5-(3-hydroxyprop-1-yny)-1,3-dihydropyrimidine-2-one, HOPC; and 6-amino-5-(3-aminoprop-1-yny)-1,3-dihydropyrimidine-2-one, NH_2PC; 5-[4-amino-3-(3-methoxyprop-1-ynyl)pyrazol[3,4-d]pyrimidinyl]-2-(hydroxymethyl)oxolan-3-ol, CH_3 OPPPA; 6-amino-1-[4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-3-(3-methoxyprop-1-ynyl)-5-hydropyrazolo[3,4-d]pyrimidin-4-one, CH_3 OPPPG; 4, (4,6-Diamino-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-but-3-yn-1-ol, Super A; 6-Amino-3-(4-hydroxy-but-1-ynyl)-1,5-dihydro-pyrazolo[3,4-d]pyrimidin-4-one; 5-(4-hydroxy-but-1-ynyl)-1H-pyrimidine-2,4-dione, Super T; 3-iodo-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine ($(NH_2)_2$PPAI); 3-bromo-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine ($(NH_2)_2$ PPABr); 3-chloro-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine ($(NH_2)_2$PPACl); 3-Iodo-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (PPAI); 3-Bromo-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (PPABr); and 3-chloro-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (PPACl). The term encompasses base analogs that provide alternative hydrogen bonding configurations (*e.g.,* Iso-C and Iso-G and other non-standard base pairs described in U.S. Patent No. 6,001,983 to S. Benner); non-hydrogen bonding analogs (*e.g.,* non-polar, aromatic nucleoside analogs such as 2,4-difluorotoluene, described by B.A. Schweitzer and E.T. Kool, J. Org. Chem., 1994, 59, 7238-7242, B.A. Schweitzer and E.T. Kool, J. Am. Chem. Soc., 1995, 117, 1863-1872); "universal" bases such as 5-nitroindole and 3-nitropyrrole; and universal purines and pyrimidines (such as

"K" and "P" nucleotides, respectively; P. Kong, *et al*., Nucleic Acids Res., 1989, 17, 10373-10383, P. Kong *et al*., Nucleic Acids Res., 1992, 20, 5149-5152). Nucleotide analogs also include nucleotides and polynucleotides having one or more modified or alternative sugar and/or phosphate moieties, including but not limited to phosphorothioate nucleotides, phosphonate nucleotides, 2' substituted sugars (*e.g.*, 2'-O-methyl ribose) and/or alternative sugars (*e.g.*, arabinose). Nucleotide analogs include modified forms of deoxyribonucleotides as well as ribonucleotides.

A nucleic acid sequence or molecule may be DNA or RNA, of either genomic or synthetic origin, that may be single or double stranded, and represent the sense or antisense strand. Thus, nucleic acid sequence may be dsDNA, ssDNA, mixed ssDNA, mixed dsDNA, dsDNA made into ssDNA (*e.g.,* through melting, denaturing, helicases, *etc.*), A-, B-, or Z-DNA, triple-stranded DNA, RNA, ssRNA, dsRNA, mixed ss and dsRNA, dsRNA made into ssRNA (*e.g.,* via melting, denaturing, helicases, *etc.*), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), catalytic RNA, snRNA, microRNA, or protein nucleic acid (PNA).

The present technology is not limited by the type or source of nucleic acid (*e.g.,* sequence or molecule (*e.g.,* target sequence and/or oligonucleotide)) utilized. For example, the nucleic acid sequence may be amplified or created sequence (*e.g.,* amplification or creation of nucleic acid sequence via synthesis (*e.g.,* polymerization (*e.g.,* primer extension (*e.g.,* RNA-DNA hybrid primer technology)) and reverse transcription (*e.g.,* of RNA into DNA)) and/or amplification (*e.g.,* polymerase chain reaction (PCR), rolling circle amplification (RCA), nucleic acid sequence based amplification (NASBA), transcription mediated amplification (TMA), ligase chain reaction (LCR), cycling probe technology, Q-beta replicase, strand displacement amplification (SDA), branched-DNA signal amplification (bDNA), hybrid capture, and helicase dependent amplification).

The terms “nucleotide” and “base” are used interchangeably when used in reference to a nucleic acid sequence, unless indicated otherwise herein, and refer to a nucleoside comprising a sugar molecule and a nucleobase, and optionally a 5′ phosphate. A “nucleobase” is a heterocyclic nitrogenous base such as adenine, guanine, cytosine, thymine, uracil, inosine, xanthine, hypoxanthine, or a heterocyclic derivative, analog, or tautomer thereof. A nucleobase can be

naturally occurring or synthetic. Non-limiting examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, xanthine, hypoxanthine, 8-azapurine, purines substituted at the 8 position with methyl or bromine, 9-oxo-N6-methyladenine, 2-aminoadenine, 7-deazaxanthine, 7-deazaguanine, 7-deaza-adenine, N4-ethanocytosine, 2,6-diaminopurine, N6-ethano-2,6-diaminopurine, 5-methylcytosine, 5-(C3-C6)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, thiouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridine, isocytosine, isoguanine, inosine, 7,8-dimethylalloxazine, 6-dihydrothymine, 5,6-dihydrouracil, 4-methyl-indole, ethenoadenine and the non-naturally occurring nucleobases described in U.S. Pat. Nos. 5,432,272 and 6,150,510 and PCT applications WO 92/002258, WO 93/10820, WO 94/22892, and WO 94/24144, and Fasman ("Practical Handbook of Biochemistry and Molecular Biology", pp. 385-394, 1989, CRC Press, Boca Raton, LO), all herein incorporated by reference in their entireties.

As used herein, the term "non-standard" as used in reference to nucleotides and nucleobases, *e.g.,* in oligonucleotides, refers to nucleotides other than adenine, guanine, cytosine, thymine, and uracil deoxy- and ribonucleotides. Non-standard nucleotides include analog and derivative nucleobases, including but not limited to those described hereinabove in reference to "nucleic acid sequence" and "nucleotide" definitions.

As used herein, the term "standard" as used in reference to nucleotides and nucleobases, *e.g.,* in oligonucleotides, refers to adenine, guanine, cytosine, thymine, and uracil deoxy- and ribonucleotides.

The term "oligonucleotide" as used herein is defined as a molecule comprising two or more nucleotides (*e.g.,* deoxyribonucleotides or ribonucleotides), preferably at least 5 nucleotides, more preferably at least about 10–15 nucleotides and more preferably at least about 15 to 30 nucleotides, or longer (*e.g.,* oligonucleotides are typically less than 200 residues long (*e.g.,* between 15 and 100 nucleotides), however, as used herein, the term is also intended to encompass longer polynucleotide chains). The exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide. Oligonucleotides are often referred to by their length. For example, a 24-residue oligonucleotide is referred to as a "24-mer". Oligonucleotides can form secondary and tertiary structures by self-hybridizing or by hybridizing to other polynucleotides. Such structures can include, but are not limited to,

duplexes, hairpins, cruciforms, bends, and triplexes. Oligonucleotides may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription, PCR, or a combination thereof. In some embodiments, oligonucleotides that form invasive cleavage structures are generated in a reaction (*e.g.,* by extension of a primer in an enzymatic extension reaction).

Because mononucleotides are reacted to make oligonucleotides in a manner such that the 5′ phosphate of one mononucleotide pentose ring is attached to the 3′ oxygen of its neighbor in one direction via a phosphodiester linkage, an end of an oligonucleotide is referred to as the "5′ terminus" or 5′ end if its 5′ phosphate is not linked to the 3′ oxygen of a mononucleotide pentose ring and as the "3′ terminus or 3′ end" if its 3′ oxygen is not linked to a 5′ phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5′ and 3′ ends. As distinct from the 3′ and 5′ ends of a nucleic acid molecule, the 3′ and 5′ ends of a nucleic acid sequence need not be at the 3′ and 5′ termini of the molecule strand. A first region along a nucleic acid strand is said to be upstream of another region if the 3′ end of the first region is before the 5′ end of the second region when moving along a strand of nucleic acid in a 5′ to 3′ direction.

When two different, non-overlapping oligonucleotides anneal to different regions of the same linear complementary nucleic acid sequence, and the 3′ end of one oligonucleotide points towards the 5′ end of the other, the former may be called the "upstream" oligonucleotide and the latter the "downstream" oligonucleotide.

As used herein, "base pairing rules" refers to the rules for the pairing of nucleotide bases established by Watson and Crick. In standard Watson-Crick geometry, large purines (A and G bases) base pair with a small pyrimidine bases (T and C), with A pairing with T (or U) and G pairing with C, so that the A-T base pair is essentially the same size as the G-C base pair. This means that the rungs of a DNA ladder, formed from either A-T (or A-U) or G-C base pairs, all have the same length across the helix. Under the standard Watson-Crick rules for base pairing, A-T and A-U base pairs form two hydrogen bonds between the base moieties, and G-C base pairs form three hydrogen bonds.

Nucleotides may pair in non-standard ways, such as in "wobble" base pairing, in which one or more hydrogen bonds are formed but the geometry of the base pair does not fit within the

standard Watson-Crick geometry. Among the most common of the wobble base pairs are G-T (or U) base pairing, in which a single hydrogen bond is formed between G and T (or G and U). Non-Watson–Crick base pairing also includes base pairs formed by inosine (I) with C, T, or A, purine base pairs A-G (or I), A-A, and G- C, and the reverse Watson–Crick base pair A-T, in which the T ring is rotated 180° from the normal Watson–Crick pair. See, *e.g.,* Hyone-Myong Eun, *Enzymology Primer for Recombinant DNA Technology,* Academic Press, (1996).

As used herein, "standard base pair/base pairing" refers to base pairs having standard Watson-Crick geometry, and encompassing A-T, A-U, and G-C base pairs. The term encompasses base pairing of modified analogs of these nucleotides in which the modification (*e.g.,* an attached dye or other moiety) does not substantially alter the hydrogen bonding between the bases (*e.g.,* the number, positions, or acceptor/donor pairs of the hydrogen bonds.) As used herein, "non-standard base pair/base pairing" refers to pairing between nucleotides *e.g.,* natural nucleotides, nucleotide analogs, that does not fall within standard base pairing having standard Watson-Crick geometry.

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (*e.g.,* a sequence of two or more nucleotides (*e.g.,* an oligonucleotide or a target nucleic acid)) related by the base-pairing rules. For example, the sequence "5′-A-G-T-3′," is complementary to the sequence "3′-T-C-A-5′." Complementarity may be "partial," in which only some of the nucleic acid bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acid bases. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods that depend upon the association of two or more nucleic acid strands. Either term may also be used in reference to individual nucleotides, especially within the context of polynucleotides. For example, a particular nucleotide within an oligonucleotide may be noted for its complementarity, or lack thereof, to a nucleotide within another nucleic acid sequence (*e.g.,* a target sequence), in contrast or comparison to the complementarity between the rest of the oligonucleotide and the nucleic acid sequence.

The complement of a nucleic acid sequence as used herein refers to an oligonucleotide which, when aligned with the nucleic acid sequence such that the 5′ end of one sequence is

paired with the 3′ end of the other, is in "antiparallel association." Nucleotide analogs, as discussed above, may be included in the nucleic acids of the present technology, and include, for example, inosine, 7-deazaguanine, iso-C, and iso-G. Complementarity need not be perfect; stable duplexes may contain mismatched base pairs or unmatched bases. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length of the oligonucleotide, base composition, and sequence of the oligonucleotide, ionic strength, and incidence of mismatched base pairs.

As used herein, the term "label" refers to any moiety (*e.g.,* chemical species) that can be detected or can lead to a detectable response. In some preferred embodiments, detection of a label provides quantifiable information. Labels can be any known detectable moiety, such as, for example, a radioactive label (*e.g.,* radionuclides), a ligand (*e.g.,* biotin or avidin), a chromophore (*e.g.,* a dye or particle that imparts a detectable color), a hapten (*e.g.,* digoxygenin), a mass label, latex beads, metal particles, a paramagnetic label, a luminescent compound (*e.g.,* bioluminescent, phosphorescent or chemiluminescent labels) or a fluorescent compound. A label may also comprise a phosphor particle for detection. In particularly preferred embodiments, the phosphor particle is an up-converting phosphor particle (see, *e.g.,* Ostermayer, F.W. Preparation and properties of infrared-to-visible conversion phosphors. Metall.Trans. 752, 747–755 [1971]). In some embodiments, rare earth-doped ceramic particles are used as phosphor particles. Phosphor particles may be detected by any suitable method, including but not limited to up-converting phosphor technology (UPT), in which up-converting phosphors transfer low energy infrared (IR) radiation to high-energy visible light. While the present invention is not limited to any particular mechanism, in some embodiments the UPT up-converts infrared light to visible light by multi-photon absorption and subsequent emission of dopant-dependent phosphorescence. See, *e.g.,* U.S. Patent No. 6,399,397, Issued June 4, 2002, to Zarling, *et al.*; van De Rijke, *et al.*, Nature Biotechnol. 19(3):273-6 [2001]; Corstjens, *et al.*, IEE Proc. Nanobiotechnol. 152(2):64 [2005], each incorporated by reference herein in its entirety.

As used herein, the term "distinct" in reference to signals, *e.g.,* from different labels, refers to signals that can be differentiated one from another, *e.g.,* by spectral properties such as fluorescence emission wavelength, color, absorbance, mass, size, fluorescence polarization

properties, charge, *etc.,* or by capability of interaction with another moiety, such as with a chemical reagent, an enzyme, an antibody, *etc.*

A label may be joined, directly or indirectly, to an oligonucleotide or other biological molecule. Direct labeling can occur through bonds or interactions that link the label to the oligonucleotide, including covalent bonds or non-covalent interactions such as hydrogen bonding, hydrophobic and ionic interactions, or through formation of chelates or coordination complexes. Indirect labeling can occur through use of a bridging moiety or "linker," such as an antibody or additional oligonucleotide(s), which is/are either directly or indirectly labeled.

Labels can be used alone or in combination with moieties that can suppress (*e.g.,* quench), excite, or transfer (*e.g.,* shift) emission spectra (*e.g.,* fluorescence resonance energy transfer (FRET)) of a label (*e.g.,* a luminescent label).

A "polymerase" is an enzyme generally for joining 3′-OH 5′-triphosphate nucleotides, oligomers, and their analogs. Polymerases include, but are not limited to, template-dependent DNA-dependent DNA polymerases, DNA-dependent RNA polymerases, RNA-dependent DNA polymerases, and RNA-dependent RNA polymerases. Polymerases include but are not limited to T7 DNA polymerase, T3 DNA polymerase, T4 DNA polymerase, T7 RNA polymerase, T3 RNA polymerase, SP6 RNA polymerase, DNA polymerase 1, Klenow fragment, *Thermophilus aquaticus* DNA polymerase, *Tth* DNA polymerase, Vent DNA polymerase (New England Biolabs), Deep Vent DNA polymerase (New England Biolabs), *Bst* DNA Polymerase Large Fragment, Stoeffel Fragment, 9°N DNA Polymerase, *Pfu* DNA Polymerase, *Tfl* DNA Polymerase, RepliPHI *Phi29* Polymerase, *Tli* DNA polymerase, eukaryotic DNA polymerase beta, telomerase, Therminator polymerase (New England Biolabs), KOD HiFi DNA polymerase (Novagen), KOD1 DNA polymerase, Q-beta replicase, terminal transferase, AMV reverse transcriptase, M-MLV reverse transcriptase, *Phi6* reverse transcriptase, HIV-1 reverse transcriptase, novel polymerases discovered by bioprospecting, and polymerases cited in US 2007/0048748, U.S. Pat. Nos. 6,329,178; 6,602,695; and 6,395,524 (incorporated by reference). These polymerases include wild-type, mutant isoforms, and genetically engineered variants.

A "DNA polymerase" is a polymerase that produces DNA from deoxynucleotide monomers (dNTPs). "Eubacterial DNA polymerase" as used herein refers to the Pol A type DNA polymerases (repair polymerases) from Eubacteria, including but not limited to DNA

Polymerase I from *E. coli*, Taq DNA polymerase from *Thermus aquaticus* and DNA Pol I enzymes from other members of genus *Thermus*, and other eubacterial species *etc.*

As used herein, a nucleic acid "ligase" refers to an enzyme capable of catalyzing the formation of a covalent bond between a 5′ end of a nucleic acid strand and a 3′ end of a nucleic acid strand. In some embodiments, a ligase catalyzes formation of a phosphodiester bond between a 3′ hydroxyl group and a 5′-phosphoryl group, *e.g.*, at a nick in a strand of duplexed nucleic acid. Ligases may catalyze bond formation of other substrates. For example, *E. coli* RtcB ligase joins single-stranded RNA or DNA with a 3′-phosphate or a 2′,3′-cyclic phosphate to an 5′-OH of single-stranded RNA. Exemplary ligases include, *e.g.*, T4 DNA Ligase, T7 DNA Ligase, T4 RNA ligase, Taq DNA Ligase, Electroligase® T4 DNA ligase (NEB); HiFi Taq DNA Ligase (NEB), Hi-T4™ DNA ligase (NEB), Salt-T4® DNA Ligase (NEB), SplintR® Ligase (also known as PBCV-1 DNA Ligase or *Chlorella* virus DNA Ligase; NEB), *E. coli* RtcB ligase; CIRCLIGASE ssDNA ligase, CIRCLIGASE II ssDNA ligase, and AMPLIGASE Thermostable ligase.

As used herein, the term "target," as used in reference to a nucleic acid strand or site on a nucleic acid strand, refers to a nucleic acid species or nucleic acid sequence or structure sought to be sorted out from other nucleic acids, *e.g.,* by probe binding, amplification, isolation, capture, *etc.* For example, when used in reference to the polymerase chain reaction, "target" refers to the region of nucleic acid bounded by the primers used for polymerase chain reaction, while when used in an assay in which target DNA is not amplified, *e.g.,* in some embodiments of an invasive cleavage assay, a target comprises the site at which a probe and invasive oligonucleotides (*e.g.,* INVADER oligonucleotide) bind to form an invasive cleavage structure, such that the presence of the target nucleic acid can be detected. A "segment" is defined as a region of nucleic acid within the target sequence. As used in reference to a double-stranded nucleic acid, the term "target" is not limited to a particular strand of the duplexed target, *e.g.,* a coding strand, but may be used in reference to either one or both strands of, for example, a double-stranded gene or reference DNA.

Accordingly, as used herein, "non-target," *e.g.,* as it is used to describe a nucleic acid such as a DNA or RNA, refers to nucleic acid that may be present in a reaction, but that is not the subject of detection or characterization by the reaction. In some embodiments, non-target

nucleic acid may refer to nucleic acid present in a sample that does not, *e.g.,* contain a target sequence, while in some embodiments, non-target may refer to exogenous nucleic acid, *i.e.,* nucleic acid that does not originate from a sample containing or suspected of containing a target nucleic acid, and that is added to a reaction, *e.g.,* to normalize the activity of an enzyme (*e.g.,* polymerase) to reduce variability in the performance of the enzyme in the reaction.

As used herein, the terms "cell-free" and "circulating cell-free" as used in reference to nucleic acids from blood are used interchangeably and refer to nucleic acids, *e.g.,* DNA and RNA species, that are found in blood but that are not within cells in the blood. The terms as used herein with respect to nucleic acid extracted from blood refer to the nature and location of the nucleic acid *prior* to collection of the sample from the subject and prior to extraction of the nucleic acid from the blood sample.

The term "marker," as used herein, refers to a substance (*e.g.,* a nucleic acid, or a region of a nucleic acid, or a protein) that may be used to distinguish non-normal cells (*e.g.,* diseased cells or cancer cells) from normal cells (non-diseased cells or non-cancerous cells), *e.g.,* based on presence, absence, or status (*e.g.,* methylation state) of the marker substance. As used herein "normal" methylation of a marker refers to a degree of methylation typically found in normal cells, *e.g.,* in non-diseased cells or non-cancerous cells.

As used herein, the term "amplification reagents" refers to those reagents (deoxyribonucleoside triphosphates, buffer, *etc.*), needed for amplification except for primers, nucleic acid template, and the amplification enzyme. Typically, amplification reagents along with other reaction components are placed and contained in a reaction vessel.

A "reaction mixture" is a mixture of reagents (*e.g.,* oligonucleotides, target nucleic acids, enzymes, *etc.*) in a combination and/or locus in which a reaction can occur, *e.g.,* a mixture of reagents in a single reaction vessel, at a locus in a fluidic device, at locus on a surface, *etc.*

As used herein, a "multiplex" reaction is a reaction (*e.g.,* RCA, RCT, ligation, PCR, PCR-flap assay) that operates on multiple targets (2, 3, 4, 5, 6, 7, 8, ... 20, 30, 100, 1000, 100,000, *etc.*) in a single reaction mixture. Multiplexed reactions are distinguished from reactions that operate on one target analyte per reaction mixture. As used herein, the term "highly multiplexed" refers to reactions that operate on at least 6, preferably at least 10, more

preferably at least 20 or more different targets (*e.g.,* different genes, or regions of genes) in a single reaction mixture.

As used herein, the term "nested" as used in reference to analysis of target nucleic acids using sequential rounds of specific PCR refers to a second (or subsequent) round of amplification of a target nucleic acid conducted using a primer pair in which one or both of the primers in the second primer pair anneals to a site in the target sequence that is inside the region defined by the initial primer pair, such that the second primer pair is considered to be "nested" within the first primer pair. When only one of the primers in a second primer pair anneals to a site inside the region defined by the initial primer pair, the second amplification reaction is said to be "semi-nested." The terms "nested" and "semi-nested" may be used in reference to the second (or subsequent) primer pair (*e.g.*, "nested primers") and in reference to an amplification reaction performed with nested primers (*e.g.*, a "nested PCR-flap assay").

As used herein, the term "control" when used in reference to nucleic acid detection or analysis refers to a nucleic acid having known features (*e.g.,* known sequence, known copy-number per cell), for use in comparison to an experimental target (*e.g.,* a nucleic acid of unknown concentration). A control may be an endogenous, preferably invariant gene against which a test or target nucleic acid in an assay can be normalized. Such normalizing controls for sample-to-sample variations that may occur in, for example, sample processing, assay efficiency, *etc.,* and allows accurate sample-to-sample data comparison. Controls may also be external. For example, in quantitative assays such as qPCR, QuARTS, *etc.,* a "calibrator" or "calibration control" is a nucleic acid of known sequence, *e.g.,* having the same sequence as a portion of an experimental target nucleic acid, and a known concentration or series of concentrations (*e.g.,* a serially diluted control target for generation of calibration curved in quantitative PCR). Typically, calibration controls are analyzed using the same reagents and reaction conditions as are used on an experimental DNA. In certain embodiments, the measurement of the calibrators is done at the same time, *e.g.,* in the same thermal cycler, as the experimental assay. In preferred embodiments, multiple calibrators may be included in a single plasmid, such that the different calibrator sequences are easily provided in equimolar amounts. In particularly preferred embodiments, plasmid calibrators are digested, *e.g.,* with one or more restriction enzymes, to

release calibrator portion from the plasmid vector. See, *e.g.,* WO 2015/066695, which is included herein by reference.

As used herein the term "fish DNA" refers to bulk (*e.g.,* genomic) DNA isolated from fish, *e.g.,* as described in U.S. Patent No.9,212,392. Bulk purified fish DNA is commercially available, *e.g.,* provided in the form of cod and/or herring sperm DNA (Roche Applied Science, Mannheim, Germany) or salmon DNA (USB/Affymetrix). "Fish DNA" is distinct from any particular gene from a fish that is in isolated form, *e.g.,* that has been separately synthesized or that has been separated from the other DNA of the fish genome.

As used herein, the "sensitivity" of a given marker (or set of markers used together) refers to the percentage of samples that report a DNA methylation value above a threshold value that distinguishes between neoplastic and non-neoplastic samples. In some embodiments, a positive is defined as a histology-confirmed neoplasia that reports a DNA methylation value above a threshold value (*e.g.,* the range associated with disease), and a false negative is defined as a histology-confirmed neoplasia that reports a DNA methylation value below the threshold value (*e.g.,* the range associated with no disease). The value of sensitivity, therefore, reflects the probability that a DNA methylation measurement for a given marker obtained from a known diseased sample will be in the range of disease-associated measurements. As defined here, the clinical relevance of the calculated sensitivity value represents an estimation of the probability that a given marker would detect the presence of a clinical condition when applied to a subject with that condition.

As used herein, the "specificity" of a given marker (or set of markers used together) refers to the percentage of non-neoplastic samples that report a DNA methylation value below a threshold value that distinguishes between neoplastic and non-neoplastic samples. In some embodiments, a negative is defined as a histology-confirmed non-neoplastic sample that reports a DNA methylation value below the threshold value (*e.g.,* the range associated with no disease), and a false positive is defined as a histology-confirmed non-neoplastic sample that reports a DNA methylation value above the threshold value (*e.g.,* the range associated with disease). The value of specificity, therefore, reflects the probability that a DNA methylation measurement for a given marker obtained from a known non-neoplastic sample will be in the range of non-disease associated measurements. As defined here, the clinical relevance of the calculated specificity

value represents an estimation of the probability that a given marker would detect the absence of a clinical condition when applied to a patient without that condition.

As used herein, a "selected nucleotide" refers to one nucleotide of the four typically occurring nucleotides in a nucleic acid molecule (C, G, T, and A for DNA and C, G, U, and A for RNA), and can include methylated derivatives of the typically occurring nucleotides (*e.g.,* when C is the selected nucleotide, both methylated and unmethylated C are included within the meaning of a selected nucleotide), whereas a methylated selected nucleotide refers specifically to a nucleotide that is typically methylated and an unmethylated selected nucleotides refers specifically to a nucleotide that typically occurs in unmethylated form.

The term "methylation-specific restriction enzyme" refers to a restriction enzyme that selectively digests a nucleic acid dependent on the methylation state of its recognition site. In the case of a restriction enzyme that specifically cuts if the recognition site is not methylated or is hemi-methylated (a methylation-sensitive enzyme), the cut will not take place (or will take place with a significantly reduced efficiency) if the recognition site is methylated on one or both strands. In the case of a restriction enzyme that specifically cuts only if the recognition site is methylated (a methylation-dependent enzyme), the cut will not take place (or will take place with a significantly reduced efficiency) if the recognition site is not methylated. Preferred are methylation-specific restriction enzymes, the recognition sequence of which contains a CG dinucleotide (for instance a recognition sequence such as CGCG or CCCGGG). Further preferred for some embodiments are restriction enzymes that do not cut if the cytosine in this dinucleotide is methylated at the carbon atom C5.

The terms "selectively binds" and "specifically binds" (or selectively or specifically binding, bound, hybridizes, hybridizing, anneals, annealing, *etc.*) as used in reference to interaction between oligonucleotides or other nucleic acids are used herein interchangeably to refer to hybridization or base-pairing that is sufficiently sequence-selective that the oligonucleotide or nucleic acid will preferentially hybridize to a particular nucleic acid (*e.g.,* a target nucleic acid having a particular nucleotide sequence) and will not substantially bind to non-target nucleic acids (*e.g.,* having slightly or completely different nucleotide sequences than the target nucleic acid), under conditions in which selective or specific binding is conducted.

The term “probe” or “oligonucleotide probe” refers to an oligonucleotide (*e.g.,* a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly, or by PCR amplification, that is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification, and isolation of particular gene sequences (*e.g.,* a “capture probe”). It is contemplated that any probe used in the present invention may, in some embodiments, be labeled with any “reporter molecule,” so that it is detectable in any detection system, including, but not limited to enzyme (*e.g.,* ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label. Probe oligonucleotides include, *e.g.,* “flap oligonucleotides” that, when used in reference to a flap assay, refers to an oligonucleotide that interacts with a target nucleic acid to form a cleavage structure in the presence of an invasive oligonucleotide, and padlock probes that interact with a nucleic acid strand to form an invasive cleavage structure. In some embodiments, flap oligonucleotides comprise a portion, typically a 5′ portion that forms a single-stranded 5′ flap of several nucleotides in the invasive cleavage structure. As used herein in reference to invasive cleavage structures, “probe oligonucleotide” is used interchangeably with “flap oligonucleotide” and refers to the oligonucleotide that forms the downstream duplex and is cleaved in an invasive cleavage structure.

The term “invasive oligonucleotide” refers to an oligonucleotide that hybridizes to a target nucleic acid at a location adjacent to the region of hybridization between a probe and the target nucleic acid, wherein the 3′ end of the invasive oligonucleotide comprises a portion (*e.g.,* a chemical moiety, or one or more nucleotides) that overlaps with the region of hybridization between the probe and target. The 3′ terminal nucleotide of the invasive oligonucleotide may but need not be complementary with a corresponding nucleotide in the target. In some embodiments, the invasive oligonucleotide contains sequences at its 3′ end that are substantially the same as sequences located at the 5′ end of a portion of the probe oligonucleotide that anneals to the target strand. In some embodiments, *e.g.,* in a PCR-flap assay, a primer used for amplification may also serve as an invasive oligonucleotide with probe.

As used herein, the term “kit” refers to any delivery system for delivering materials. In the context of nucleic acid purification systems and reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reagents and devices (*e.g.,* chaotropic salts, particles, buffers, denaturants, oligonucleotides, filters *etc.* in the appropriate containers) and/or supporting materials (*e.g.,* sample processing or sample storage vessels, written instructions for performing a procedure, *etc.*) from one location to another. For example, kits include one or more enclosures (*e.g.,* boxes) containing the relevant reaction reagents and/or supporting materials. As used herein, the term “fragmented kit” refers to a delivery system comprising two or more separate containers that each contains a subportion of the total kit components. The containers may be delivered to the intended recipient together or separately. For example, a first container may contain materials for sample collection and a buffer, while a second container contains capture oligonucleotides and denaturant. The term “fragmented kit” is intended to encompass kits containing Analyte specific reagents (ASR's) regulated under section 520(e) of the Federal Food, Drug, and Cosmetic Act, but are not limited thereto. Indeed, any delivery system comprising two or more separate containers that each contains a subportion of the total kit components are included in the term “fragmented kit.” In contrast, a “combined kit” refers to a delivery system containing all of the components of a reaction assay in a single container (*e.g.,* in a single box housing each of the desired components). The term “kit” includes both fragmented and combined kits.

The term “system” as used herein refers to a collection of articles for use together for a particular purpose. In some embodiments, the articles comprise instructions for use, as information supplied on *e.g.,* an article, on paper, online (*e.g.,* at a website or web address) on recordable media (*e.g.,* diskette, CD, DVD, flash drive, *etc.*). In some embodiments, instructions direct a user to an online location, *e.g.,* a website for viewing, hearing, and/or downloading instructions. In some embodiments, instructions or other information are provided as an application (“app”), *e.g.,* for a computer or for a mobile device, such as a smart phone.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 provides a schematic diagram of an exemplary invasive cleavage structure formed using a flap oligonucleotide as described herein. In this embodiment, the flap oligonucleotide comprises the target-specific region and the 5′ flap sequence, and a separate oligonucleotide (*e.g.,* an invasive oligonucleotide) is added to form the invasive cleavage structure. In this embodiment, the invasive oligonucleotide is hybridized with a nucleic acid strand, *e.g.,* a target DNA, to form an upstream duplex, with a single nucleotide unpaired at the 3′ end of the invasive oligonucleotide. A downstream oligonucleotide (*e.g.,* the target-specific region of the flap oligonucleotide) is hybridized to the nucleic acid strand (*e.g.,* the target DNA) to form a downstream duplex. In the illustrated embodiment, the downstream oligonucleotide comprises an unpaired 5′ flap (*e.g.,* the flap sequence). As illustrated, the upstream and downstream duplexes are immediately adjacent to each other, such that the target strand has no gap in base pairing between the upstream and downstream duplexes. The structure shown in FIG. 1 is a substrate for cleavage by FEN-1 endonucleases, with cleavage typically occurring after the first paired base of the oligonucleotide in the downstream duplex, as illustrated. Accordingly, cleavage at the cleavage site results in cleavage of the flap sequence from the flap oligonucleotide, thereby generating a cleaved flap sequence. In this embodiment, the cleaved flap sequence comprises one additional base compared to the uncleaved flap sequence. For example, in some embodiments the flap sequence comprises 14 bases, and the cleaved flap sequence comprises 15 bases.

FIG. 2 shows a schematic diagram of another exemplary invasive structure formed using a padlock probe comprising a flap portion and an invasive portion, as described herein. In this embodiment, the flap portion acts as a flap oligonucleotide and comprises a target-specific region that hybridizes to a first portion of the target nucleic acid and a second target-specific region that hybridizes to a second portion of the target nucleic acid. For example, the exemplary flap oligonucleotide shown in FIG. 2 is hybridized to a nucleic acid strand (*e.g.,* the target nucleic acid). The flap oligonucleotide is an unligatable padlock probe comprising a target-specific 5′ region (*e.g.,* a first target-specific region) that forms a downstream duplex with the target strand, and a target-specific 3′ end (*e.g.,* a second target-specific region) that forms an adjacent upstream duplex with the target strand. In this embodiment, the first target-specific region is proximal to the flap sequence (*e.g.,* the 5′ flap sequence, or 5′ arm), as shown in the figure. The flap

oligonucleotide further comprises a connecting backbone sequence that joins the two target-specific domains. The backbone sequence is shown in FIG. 2 as a grey line. Cleavage of the structure by a FEN endonuclease removes the 5′ flap and one nucleotide of the downstream duplex (*e.g.,* one nucleotide from the first target-specific region). After cleavage, the cleaved flap sequence may be detected by any of the methods discussed herein and the remainder of the cleaved probe forms a ligatable nick, which may be ligated to form a circular structure.

FIG. 3 is a schematic showing how the methods described herein can be used to determine whether a target nucleic acid is present in a sample based upon whether cleavage of the flap sequence (*e.g.,* the 5′ arm) of a flap oligonucleotide disclosed herein occurs. In this embodiment, an invader oligonucleotide is shown as forming an invasive cleavage structure with the target sequence and the flap oligonucleotide disclosed herein, but it is understood that the flap oligonucleotide itself (as described in FIG. 2) may comprise the first and second target-specific regions such that no invasive oligonucleotide is required in order to form the invasive cleavage structure. As shown in FIG. 3, successful cleavage results in formation of a cleaved flap sequence, which generally contains one additional base (*e.g.,* contains the terminal base of the target-specific region) compared to the uncleaved flap sequence. Successful cleavage results in a cleaved flap sequence that may serve as a primer, *e.g.*, to replicate a circular template (bottom left). In contrast, no cleavage results in a flap structure that remains attached to the target-specific region, thereby preventing replication of the circular template.

FIG. 4 is a schematic showing that the flap oligonucleotides and methods of use thereof (*e.g.,* in flap assays) described herein enable multiplexed detection of a plurality of targets in a sample. Different colors (*e.g.,* gray, black, white) indicate that different sequences can be used for the flap oligonucleotides disclosed herein (*e.g.,* different sequences for the flap sequence, different sequences for the target-specific region) with equal success at generating a cleaved flap sequence and, for example, acting as a primer for extension on circular template (as shown in FIG. 4) or being used to form a closed circular structure (*e.g.*, as in the embodiments illustrated in FIGS. 5A-6C), or being used with a vector pair of oligonucleotides to form a linear vector structure (as in one embodiment illustrated in FIG. 14), which can subsequently be amplified and/or detected and/or sequenced.

FIG. 5A is a schematic showing exemplary embodiments involving suitable probes that generate templates comprising a cleaved flap sequence, if present in the sample, wherein the templates are suitable for rolling circle amplification. As shown in FIG. 5A, in some embodiments, suitable probes include padlock probes. In the embodiment shown in the figure, the invasive cleavage structure is formed using an invasive oligonucleotide and a separate flap oligonucleotide that comprises the flap sequence ("Arm 1"). However, it is understood that the invasive cleavage structure may also be formed using a flap oligonucleotide as shown in FIG. 2, containing two target-specific regions, wherein the flap portion and invasive portion of the flap oligonucleotide each bind to different but adjacent regions on the target nucleic acid to form the invasive cleavage structure. Successful cleavage at the target site releases a cleaved flap sequence, plus 1 nucleotide of the target-specific region of the flap oligonucleotide. In this exemplary embodiment using a padlock probe, the cleaved flap sequence hybridizes to a bridge oligonucleotide that also hybridized to the two ends of the padlock probe, and forming two ligatable nicks. In some embodiments, the bridge oligonucleotide comprises an inner segment that is complementary to the cleaved flap sequence, along with a 3′ end and a 5′ end that are complementary to a first target region and a second target region of a padlock probe. However, when the flap oligonucleotide is intact (*e.g.,* when the flap is not cleaved from the flap oligonucleotide) the flap sequence of the oligonucleotide hybridizes to the bridge oligonucleotide to form only one ligatable nick and the flap cannot be ligated to the padlock probe to form a circularized molecule.

FIG. 5B shows another exemplary embodiment using a dumbbell probe. The dumbbell probe comprises two stem loop structures separated by a single-stranded region complementary to a cleaved flap sequence (termed a "bridge sequence"). In some embodiments, a closed circular structure is not properly formed until successful ligation of both ends of the cleaved flap sequence to the probe. The cleaved flap sequence ("Arm 1" plus 1 nucleotide of the target-specific region of the flap oligonucleotide) hybridizes to the bridge sequence and forms ligatable nicks. However, when the flap oligonucleotide is intact (*e.g.,* when the flap sequence is not cleaved from the flap oligonucleotide) the flap sequence of the oligonucleotide hybridizes to the bridge sequence, but the flap cannot be ligated to form a circularized dumbbell probe.

FIG. 5C shows another exemplary embodiment using a vector pair comprising two vector oligonucleotides. In the embodiment shown in the figure, the invasive cleavage structure is formed using an invasive oligonucleotide and a separate flap oligonucleotide that comprises the flap sequence ("Arm 1"). Successful cleavage at the target site releases a cleaved flap sequence, plus 1 nucleotide of the target-specific region of the flap oligonucleotide. In this exemplary embodiment using a vector pair, the cleaved flap sequence hybridizes to a bridge oligonucleotide that also hybridized to each of the two oligonucleotides of the vector pair (Vector1 and Vector2), and forming two ligatable nicks. In some embodiments, the bridge oligonucleotide comprises an inner segment that is complementary to the cleaved flap sequence, along with a 3′ end and a 5′ end that are complementary to a first target region in a first vector oligonucleotide and a second target region in the second vector oligonucleotide. Upon ligation, a linear ligated product is formed, comprising the first vector oligonucleotide, the cleaved flap, and the second vector oligonucleotide. However, when the flap oligonucleotide is intact (*e.g.,* when the flap is not cleaved from the flap oligonucleotide) the flap sequence of the oligonucleotide hybridizes to the bridge oligonucleotide to form only one ligatable nick and the flap can be ligated to only one oligonucleotide of the vector pair (*e.g.*, Vector1 in the embodiment shown).

FIG. 6A is a schematic showing that ligation of the nicks formed with the cleaved flap sequence, as shown in FIGS. 5A and 5B, results in formation of a closed circular structure that serves as a template for rolling circle amplification. For the embodiment using the padlock probe, ligation of the nicks results in proper formation of a closed circular structure, which can subsequently be detected. For the embodiment using the dumbbell probe, ligation of the nicks results in the formation of a closed circular structure in the form of a loop-stem-loop structure that serves as a suitable template for, for example, PCR, RCA, RCT, sequencing, *etc.*

FIG. 6B is a schematic showing embodiments comprising intrastrand ligation of the ends of a cleaved flap sequence (cleaved Arm 1). In one embodiment (left), a splint oligonucleotide is used to align the 5′ and 3′ ends of the cleaved flap to form a nick that is ligatable by a DNA ligase, *e.g.*, HiFi Ampligase, T4 DNA ligase, *etc.* In another embodiment (right), a single strand ligase (*e.g.*, T4 RNA ligase, CircLigase™ ssDNA ligase) is used to ligate the 5′ and 3′ ends of the cleaved flap to each other without use of a splint oligonucleotide. In both embodiments, intrastrand ligation of the ends of the cleaved flap results in formation of a closed circular

molecule that serves as a suitable template for subsequent detection, *e.g.*, PCR, RCA, RCT, sequencing, *etc.*

FIG. 6C is a schematic diagram illustrating an embodiment in which hybridization of a cleaved flap to a splint oligonucleotide does not produce a ligatable nick but instead produces an overlap cleavage structure. Cleavage to remove the flap, *e.g.*, with a flap endonuclease, produces a ligatable nick. Ligation of the ends of the cleaved flap results in formation of a closed circular molecule that serves as a suitable template for subsequent detection, *e.g.*, PCR, RCA, RCT, sequencing, *etc.*

FIG. 7A is a schematic showing preamplification steps performed prior to conducting a cleavage assay. As shown in the schematic, multiple targets can be amplified, thus generating a library of different amplicons. The amplicons can be subjected to a cleavage assay and subsequent detection, for instance, through PCR, RCA, RCT, or sequencing steps.

FIG. 7B is a schematic showing an embodiment of preamplification in which one of the target-specific primers in the preamplification reaction comprises a universal tag sequence that is the same for multiple different targets. After the preamplification step, the PCR-flap assay is performed using one original target-specific primer and probe and a universal primer. As with the embodiment shown in FIG. 7A, multiple different targets can be preamplified together in this embodiment, thus generating a library of different amplicons. The amplicons can be subjected to a flap cleavage assay and subsequent detection, for instance, through PCR, RCA, RCT, or sequencing steps.

FIG. 7C is a schematic showing an embodiment of preamplification in which one of the target-specific primers in the preamplification reaction comprises a universal tag sequence that is the same for multiple different targets. After the preamplification step, the PCR-flap assay is performed using a nested target-specific primer and probe and a universal primer. As with the embodiment shown in FIG. 7A, multiple different targets can be preamplified together in this embodiment, thus generating a library of different amplicons. The amplicons can be subjected to a flap cleavage assay and subsequent detection, for instance, through PCR, RCA, RCT, or sequencing steps.

FIG. 8 is a schematic diagram of an exemplary circularizable molecule complexed with a flap-specific bridge oligonucleotide hybridized to a cleaved flap to form two ligatable nicks. The

circularizable molecule comprises an internal spacer (iSp18) to terminate any polymerization around the circularized probe. The hybridization arm (referred to as "Hyb arm") is the region where the bridge oligonucleotide and circularizable molecule are complementary. Ad1 and Ad2 are adapter sequences for circularized-specific amplification. The unique molecular identifier (UMI) is used for quantifying unique ligation events. The sample index is used for sample-specific barcoding to provide additional indexing capacity, allowing a higher level of multiplexing samples analyzed in a single sequencing run.

FIG. 9A is a schematic showing an exemplary method described herein for generation and subsequent detection of target-specific cleaved flap sequences. Target-specific flap cleavage has single base resolution and can be used for, but not limited to, the detection of single nucleotide variants, single nucleotide polymorphisms, or methylation-specific converted sequence.

FIG. 9B is a schematic showing a second exemplary method described herein for generation and subsequent detection of target-specific cleaved flap sequences.

FIG. 10 is a graph demonstrating that CIP/PNK addition following PCR-flap assay-based generation of cleaved flap sequences improves assay sensitivity, determined by methylated DNA marker reads during sequencing. Similar methods of reducing carryover polymerase activity following the PCR-flap assay include, but are not limited to, incubation with a proteinase, ligation performed under low temperature, or engineering the bridge oligonucleotide to have a nucleotide modification that inhibits DNA polymerase activity.

FIG. 11 is a bar graph demonstrating that exonuclease addition following PCR-flap assay-based ligation of cleaved flap sequences into a circularized probe reduces non-specific assay signal. Hashed bars represent conditions with no exonuclease addition. Open bars represent conditions with exonuclease treatment.

FIGS. 12A-12C provide images demonstrating the performances of different DNA ligases tested on cleaved flaps produced in an LQAS PCR-flap assay performed without a FRET cassette, as described in Example 2. FIG. 12A provides a bioanalyzer pseudogel of the final product after cleaved flap oligonucleotide ligation and circularized probe amplification enrichment.

FIG. 12B provides a graph comparing background reads generated in the no ligase control ((-) Ligase) and no-template control (TE) reactions using different DNA ligases.

FIG. 12C shows the difference in sequencing quality (generated using an Illumina MiSeq instrument) based on the DNA ligase used.

FIG. 13 is a bar graph demonstrating the correlation of splint (bridge) oligonucleotide concentration with background reads.

FIG. 14 provides a schematic diagram showing a chimeric bridge oligonucleotide that comprises a 2′-O-methyl RNA 3′ portion and a DNA 5′ portion. The bridge oligonucleotide is shown as it would hybridize to a complementary 3′ end of a first vector ("Vector1") of a vector pair to form a first nick with a cleaved flap ("cFLAP"). Use of RNA in this portion of the bridge oligonucleotide prevents extension of 3′ end of the first vector by Taq DNA polymerase carried over from the PCR-flap reaction. The 5′ portion of a second vector ("Vector2") of the vector pair is complementary to a DNA portion of the chimeric bridge oligonucleotide, and comprises an activation tail with an un-ligatable 5′ OH group. When the 5′ portion of Vector2 that has the 5′ activation tail is hybridized to the bridge oligonucleotide adjacent to the 3′ end of the cleaved flap, an invasive cleavage structure forms that is cleaved by a flap endonuclease, *e.g.*, FEN-1 endonuclease carried over from the PCR-flap amplification, releasing the activation tail and forming a second nick. The first nick and the second nick are both ligatable using, *e.g.*, SPLINTR DNA ligase. In the embodiment illustrated, Vector1 and Vector2 are provided as separate oligonucleotides in a vector pair, and ligation produces a linear ligated product. In some embodiments, first and second vector sequences may be provided on the two ends of a single circularizable molecule, such that ligation of both nicks forms a circularized molecule.

FIG. 15 provides a schematic diagram of a chimeric bridge oligonucleotide hybridized to the 3′ and 5′ portions of a vector pair and to a cleaved flap ("cFlap"). As described above, the 5′ terminus of the flap vector (Vector2) comprises an unligatable 5′ OH group and includes an activation tail that forms a cleavable structure with the cFlap. The right panel shows different possible designs for the 5′ portion of the Vector2 flap vector, for use with bridge oligonucleotides having universal hybridization arms or with bridge oligonucleotides having base-specific hybridization arms. The different Vector2 designs referred to as "*Activated; Universal Arm" represents a mixture of four different vectors each having the same universal

hybridization arm and each having a different base in the activation tail (*e.g.*, A, G, C, or T). In the Universal Hybridization Arm panel, the bridge oligonucleotide comprises a T nucleotide that base-pairs with a 3′ terminal A nucleotide in a cleaved flap. This terminal A more efficiently prompts cleavage of an activation flap that has a base-paired A nucleotide 5′ of the cleavage site in the activation flap (the "A" within the activation flap in the top structure in this panel). The other Vector2 activation flaps (*e.g.*, with G, C, or T in the activation flap) are also cleaved but at reduced efficiency. While not being limited to any particular mechanism of action, cleavage may be reduced because the G, C, and T nucleotides 5′ of the cleavage site in the activation flap do not form standard base-pairs with the T nucleotide at the corresponding position in the bridge oligonucleotide.

The Base-Specific Hybridization Arm panel of Fig. 15 illustrates an embodiment in which the Vector2 designs each have different base-specific hybridization arms, such that the activation tail of each Vector2 region, including the nucleotide 5′ of the cleavage site, specifically hybridizes to the corresponding sequence in the bridge oligonucleotide. This concept is further depicted in FIG. 16.

FIG. 16 is a schematic diagram depicting cleavage when using a mixture of four different Vector2 designs (*e.g.* having A, G, C, or T in the activation tail) used with the same universal hybridization arm sequence (left panel), or a mixture of four different Vector2 designs (*e.g.* having A, G, C, or T in the activation tail) each having a different base-specific hybridization arm. For the universal sequence design, as illustrated in the left panel, each universal Vector2 region will hybridize to the universal portion of the bridge oligonucleotide. As described above, the universal Vector2 region having an A within the activation tail, being base paired to the T shown in the universal bridge oligonucleotide, will be more efficiently cleaved, as compared to the other G, C, or T versions of the universal Vector2 design. For the base-specific Vector2 sequence designs, as illustrated in the right panel, each Vector2 portion specifically hybridizes to a bridge oligonucleotide having a matched complementary hybridization arm. As discussed above, complementarity between the base-specific hybridization arm of the bridge oligonucleotide and the corresponding Vector2 oligonucleotide ensures that, in the embodiment illustrated, only the Vector2 oligonucleotide having the A within the activation tail will hybridize

to the bridge oligonucleotide having a T at the corresponding position in the bridge oligonucleotide.

FIG. 17 is a bar graph showing the number of reads generated using a Vector2 design having a universal hybridization arm, or Vector2 design having a base-specific hybridization arm. The base-specific Vector2 design, producing the preferred invasive cleavage structures for each of the 4 different base-specific activation flaps, results in increased overall cleavage efficiency and signal generation, *e.g.*, when multiplexing markers.

FIG. 18 is a diagram showing the components used in flap amplification reactions (*e.g.*, LQAS, TELQAS). Concentrations of the invading and non-invading primer are shown that result in the asymmetric amplification. Reducing the amplification efficiency of *B3GALT6* can be accomplished by reducing the concentration(s) of primers (invasive oligonucleotides, non-invasive oligonucleotides) and/or probes (*e.g.*, FLAP oligonucleotides) in either preamplification, TELQAS, or LQAS.

FIG. 19 shows FLAP-Seq reads (y-axis) compared to TELQAS strands (x-axis) from 40 clinical samples. Cancer-specific markers are shown in closed circles and *B3GALT6* is shown in open triangles. The cancer-specific methylated DNA markers (MDMs) fall within the linear range of the FLAP-Seq assay. *B3GALT6* comparably has much higher strand count in cfDNA, and falls outside the linear range of FLAP-Seq.

FIG. 20 shows multiplexed amplification curves of a cancer-specific MDM without primer titration (grey curves) and a universal MDM reference DNA (*B3GALT6*) with primer titration (black curves). Titration of the universal MDM primer results in a reduction in amplification efficiency, resulting in the right-shifting of the amplification curves. Cancer-specific MDM amplification is not affected by titration of the primer for the universal MDM.

FIG. 21 shows FLAP-Seq reads (y-axis) compared to TELQAS strands (x-axis) from 40 clinical samples. Cancer-specific MDM markers are shown as closed circles and *B3GALT6* reference DNA is shown in open triangles. Reduced primer concentrations were used to amplify *B3GALT6.* The cancer-specific MDMs and *B3GALT6* results fall within the linear range of the FLAP-Seq assay.

DETAILED DESCRIPTION OF THE INVENTION

Provided herein is technology relating to flap oligonucleotides, in particular to methods of analyzing cleaved flaps produced by target-dependent cleavage of flap oligonucleotides by a flap endonuclease. Embodiments of the technology provide methods and compositions for multiplexed generation of cleaved flaps and for counting cleaved flaps indicative of the presence of particular target nucleic acids, *e.g.,* in a mixture of different target and non-target nucleic acids. In some embodiments, the technology provides methods of differentiating and counting cleaved flaps associated with multiple different target nucleic acids, wherein the cleaved flaps are produced in a single cleavage reaction mixture. In particular embodiments, counting the cleaved flaps comprises sequencing the cleaved flaps, *e.g.,* using a next generation sequencing method.

In this detailed description of the various embodiments, for purposes of explanation, numerous specific details are set forth to provide a thorough understanding of the embodiments disclosed. One skilled in the art will appreciate, however, that these various embodiments may be practiced with or without these specific details. In other instances, structures and devices are shown in block diagram form. Furthermore, one skilled in the art can readily appreciate that the specific sequences in which methods are presented and performed are illustrative and it is contemplated that the sequences can be varied and still remain within the spirit and scope of the various embodiments disclosed herein.

Target nucleic acids

The technology finds application in assaying any kind of target nucleic acid, *e.g.,* DNA, RNA, *etc.* In preferred embodiments, target nucleic acids comprise DNA, including but not limited to cDNA, amplified DNA, genomic DNA, circulating cell-free DNA, *etc.* In some embodiments the DNA is treated with a methylation-specific reagent, as defined hereinabove. For example, in some embodiments, target DNA (*e.g.,* DNA extracted from a sample) is treated with a bisulfite reagent such that unmethylated cytosines are converted to deoxyuracils. For example, in some embodiments the target nucleic acids are cancer-specific methylated DNA markers (MDMs). In some embodiments, treated target nucleic acid strands are replicated or

amplified, *e.g.,* by PCR, prior to hybridization of the oligonucleotide or oligonucleotides that form a cleavage structure with the target nucleic acid. For example, for target DNA treated with a bisulfite reagent, the Us in the converted target DNA sequence are replaced by Ts in the replicate strands. Embodiments of the technology are equally applicable to such treated, replicated target DNA.

Similarly, sequence variations arising from other reagent treatments, *e.g.,* TAPS technology, as described above, and sequence variations not related to methylation, *e.g.,* single nucleotide polymorphisms (SNPs), mutations, insertions, and deletions, may be assayed, *e.g.,* distinguished from reference nucleic acids, using similar design strategies.

Target nucleic acids may be prepared by any standard method for purification or isolation of nucleic acid from a source of nucleic acids, *e.g.,* from a sample. For example, kits for isolating nucleic acids from different kinds of sample, *e.g.,* environmental samples, biological samples, medical samples, *etc.,* are available from commercial suppliers, such as Qiagen, Promega Corp., and ThermoFisher Scientific.

Flap oligonucleotides

Embodiments of the technology comprise use of flap oligonucleotides having at least one target-specific region (also referred to herein as a target-specific sequence) and a flap sequence, preferably a 5′ flap sequence.

The at least one target-specific region is sufficiently complementary to a target of interest (*e.g.,* a target nucleic acid) to hybridize to the target strand under cleavage reaction conditions, whereas the flap sequence is generally an arm (*e.g.,* a 5′ arm) that is preferably not substantially complementary to the target strand of interest, such that it is not typically hybridized to the target nucleic acid, *e.g.,* under cleavage reaction conditions. Preferably, a flap sequence is long enough that, once cleaved from the flap oligonucleotide, the cleaved flap can specifically hybridize to a complementary nucleic acid strand, *e.g.,* of a ligation template, *e.g.,* a bridge oligonucleotide or FRET cassette, under conditions in which an assay to detect the cleaved flap is conducted. For example, a cleaved flap segment preferably hybridizes with suitable specificity under ligation

conditions that it selectively anneals to a matched flap-specific ligation template such that it is ligatable to an adjacent oligonucleotide hybridized to the same ligation template. In certain embodiments, the cleaved flap segment hybridizes with suitable specificity under ligation conditions that it selectively anneals to a matched flap-specific bridging sequence to form ligatable nicks with the 5′ and 3′ termini of a padlock probe, *e.g.,* as illustrated in Figs. 5A and 5B, or the 3′ terminus of Vector1 and the 5′ terminus of Vector2 oligonucleotides of the vector pair illustrated in Figs. 5C and 14. A 5′ flap sequence is generally preferably less than about 200 nucleotides, more preferably less than about 175 nucleotides in length. In some embodiments, the flap sequence is from 1 to about 150 nucleotides, preferably 1 to about 100 nucleotides, preferably about 5 to 75 nucleotides. In preferred embodiments, the 5′ flap portion is at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, or 75 nucleotides in length. In certain embodiments, the 5′ flap portion is preferably 6 to 45 nucleotides, preferably 7 to 40 nucleotides, preferably 8 to 35 nucleotides, preferably 8 to 30 nucleotides, preferably 8 to 25 nucleotides, preferably 9 to 25 nucleotides, preferably 10 to 20 nucleotides, preferably 10 to 15 nucleotides in length.

The target-specific region of a flap oligonucleotide is also not limited to any particular length or sequence. Preferably, the target-specific region of a flap oligonucleotide is selected to selectively hybridize to a particular target strand under conditions of an assay to be performed. For example, in some embodiments, the sequence is selected to form an invasive cleavage structure with a target nucleic acid and an upstream invasive oligonucleotide sequence under conditions useful or optimal for cleavage by a thermostable FEN-1 endonuclease, preferably a strict FEN-1 endonuclease, *e.g.,* at a temperature between about 25°C and 75°C, about 40°C and about 75°C, or at any temperature between, preferably between about 45°C and 70°C, preferably between about 50°C and 70°C, preferably between about 55°C, and 70°C. In preferred embodiments, flap oligonucleotide hybridizes to the target nucleic acid in the presence of a Mg^{++} buffer, preferably a high Mg^{++} buffer (*e.g.,* preferably greater than 4 mM Mg^{++}, more preferably at least about 5, 6, 7, 8, 9, 10, or 11 mM Mg^{++}.) In certain preferred embodiments, target-specific region forms a downstream duplex with the target strand that is at least 5, 6, 7, 8, 9, 10, 11, 12,

13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 base pairs in length, preferably 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 bp, preferably 7 to 15 bp, preferably 8 to 14, preferably 9 to 13, preferably 10 to 12 bp.

Invasive oligonucleotides and sequences

Embodiments of the technology comprise use of invasive oligonucleotides or invasive sequences to form FEN-1 cleavable structures with flap oligonucleotides. In some embodiments of the technology, an invasive sequence is provided on a different molecule than the flap oligonucleotide, while in some oligonucleotides, a single molecule, *e.g.,* a padlock probe, comprises a 3′ portion serving as an invasive oligonucleotide and a 5′ portion serving as a flap oligonucleotide, wherein the 3′ portion and 5′ portion are linked, preferably covalently linked, via a backbone portion of the molecule. The lengths and sequences of the 5′ and 3′ portions may be selected to selectively hybridize to particular target strands to form an invasive cleavage structure under conditions of an assay to be performed. For example, in some embodiments, the sequences are selected to form an invasive cleavage structure with a target nucleic acid under conditions useful or optimal for cleavage by a thermostable FEN-1 endonuclease, preferably a strict FEN-1 endonuclease, *e.g.,* under the temperatures and conditions discussed above for the Flap oligonucleotide. While the upstream duplex is not limited to a particular length of duplex, in certain preferred embodiments, the invasive oligonucleotide or the invasive sequence on a 3′ portion of a padlock probe forms an upstream duplex with the target (see FIG. 1) that is at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 bp long. See, *e.g.,* Kaiser M.W., *et al*. (1999) J. Biol. Chem., 274:21387.

Controls

In some embodiments, the assay utilizes a control, also referred to herein as a “reference” (*e.g.,* a “reference nucleic acid”). In some embodiments, the assay utilizes a control/reference, such as the universally-methylated DNA marker *B3GALT6*. In some embodiments, the target nucleic acids are cancer-specific methylated DNA markers, and the assay uses the control

universally methylated DNA marker *B3GALT6*. In some embodiments, the reaction mixture comprises oligonucleotides specific for the target nucleic acids (*e.g.*, flap oligonucleotides, invasive oligonucleotides, and optionally non-invasive oligonucleotides) and oligonucleotides (*e.g.*, flap oligonucleotides, invasive oligonucleotides, and optionally non-invasive oligonucleotides) specific for *B3GALT6*. In some embodiments, a concentration of one or more oligonucleotides specific for *BGALT6* is diluted relative to a concentration of an equivalent oligonucleotide for a target nucleic acid (*e.g.*, a cancer specific MDM). For example, in some embodiments the concentration of a flap oligonucleotide for *B3GALT6* is diluted relative to the concentration of the flap oligonucleotides for the target nucleic acids in the reaction mixture. As another example, in some embodiments the concentration of an invasive oligonucleotide for *B3GALT6* is diluted relative to the concentration of the invasive oligonucleotides for the target nucleic acids in the reaction mixture. As yet another example, in some embodiments the concentration of a non-invasive oligonucleotide for *B3GALT6* is diluted relative to the concentration of the non-invasive oligonucleotides for the target nucleic acids in the reaction mixture. In some embodiments, multiple oligonucleotides specific for *B3GALT6* are diluted. For example, in some embodiments one or more oligonucleotides for *B3GALT6* (*e.g.*, flap oligonucleotides, invasive oligonucleotides, non-invasive oligonucleotides) are diluted by at least 50% compared to the concentration of the equivalent oligonucleotides for the target nucleic acids. In some embodiments, the oligonucleotide(s) specific for *B3GALT6* are diluted to a concentration of 0.2, 0.1, or 0.5 relative to the equivalent concentration of the oligonucleotide(s) for the target nucleic acids.

In some embodiments an oligonucleotide (*e.g.* a FLAP oligonucleotide, an invasive oligonucleotide, a non-invasive oligonucleotide) specific for a reference nucleic acid (*e.g. B3GALT6*) binds to a region in the target nucleic acid with one or more mismatched base pairings (*e.g.* mismatched base pairings between the oligonucleotide and the target region to which it binds), thus attenuating (*e.g.* reducing) amplification efficiency of the reference nucleic acid.

Flap cleavage reactions

In some aspects, provided herein are compositions comprising a plurality of flap oligonucleotides described herein. In some embodiments, the compositions described herein find use in flap assays in which a target strand is not replicated, and in some embodiments, compositions described herein find use in amplification-flap assays in which a target strand is replicated, *e.g.,* by a polymerase. For example, in some embodiments, the flap sequence is cleaved from the flap oligonucleotide when a target nucleic acid and a suitable flap endonuclease are present in the sample and wherein the conditions are suitable for flap cleavage by the flap endonuclease, thereby generating a cleaved flap sequence. The cleaved flap sequence can subsequently be amplified and/or detected, thereby serving as a marker for the target nucleic acid in the sample. Accordingly, in some aspects provided herein are methods for multiplexed detection of a plurality of target nucleic acids in a sample, comprising contacting the sample with a composition described herein. In some embodiments a target nucleic acid is RNA, and cleavage to release a flap comprises use of a structure-specific 5′ nuclease suitable for RNA target strands, as described, *e.g.,* in WO 2003/073067 and US 7,851,150. In other embodiments, a target nucleic acid strand is DNA, and cleavage to release a flap comprises use of a flap endonuclease, preferably a FEN-1 endonuclease.

In some embodiments, provided herein is a composition comprising a plurality of flap oligonucleotides. In some embodiments, each flap oligonucleotide comprises a flap sequence and at least one target-specific region. In some embodiments, the at least one target-specific region comprises a sequence of nucleotides that binds to a target nucleic acid in the sample. In some embodiments, each flap oligonucleotide binds to a different target nucleic acid, if present in the sample. For example, in some embodiments each flap comprises a unique target-specific region, such that each target-specific region binds to a different target nucleic acid in the sample. Accordingly, the compositions described herein find use in multiplexed detection of a plurality of targets in a given sample. In some embodiments, each flap oligonucleotide comprises a single target-specific region. In some embodiments, each flap oligonucleotide comprises more than one target-specific region. For example, in some embodiments each flap oligonucleotide comprises a first target-specific region and a second target-specific region, wherein the flap portion and invasive portion of the flap oligonucleotide each bind to different but adjacent regions on the target nucleic acid to form the invasive cleavage structure, as illustrated in FIG. 2. Such a flap

oligonucleotide is particularly advantageous for forming an invasive cleavage structure without the addition of a separate invasive oligonucleotide.

An aspect of the technology is use of FEN-1 endonuclease under conditions in which the enzyme exhibits maximum specificity for an invasive cleavage structure, *e.g.,* as illustrated in FIG. 1, as compared to, *e.g.,* a gapped structure (having a single-stranded gap between the upstream and downstream duplexes), Y-structure (having a 5′ flap but no overlap between the upstream and downstream duplexes), pseudo-Y structure (having a 5′ flap and an un-duplexed template strand upstream of the downstream duplex), *etc.* Thus, in preferred embodiments, cleavage of hybridized flap oligonucleotides is done in a Mg^{++} flap assay buffer. As used herein, the term "Mg^{++} flap assay buffer" refers to a buffer solution for a flap endonuclease that includes Mg^{++} as the predominant or essentially only divalent cation in the buffer, such that cleavage reactions performed in the buffer, *e.g.,* cleavage of hybridized invasive oligonucleotide/flap oligonucleotide pairs or hybridized padlock probe that form an invasive cleavage structure, exhibits characteristics of the presence of Mg^{++}, and does not exhibit characteristics associated with the presence of other divalent cations (*e.g.,* alternative cleavage activities of flap endonucleases associated with use of Mn^{++} with or in place of Mg^{++}), as described, *e.g.,* by Kaiser, *et al*., *supra*.

In some embodiments the flap assay buffer has very low or no KCl and comprises elevated Mg^{++} as compared to standard PCR buffer, (*e.g.,* a flap assay buffer of the technology comprises >6 mM, preferably > 7mM, more preferably 7.5mM Mg^{++}, while PCR buffers typically comprise about 1.5 to 2.5 mM Mg^{++}.) In preferred embodiments, cleavage is conducted in a flap assay buffer comprising 7.5 mM $MgCl_2$, 10 mM MOPS, 0.3 mM Tris-HCl, pH 8.0, 0.8 mM KCl, 0.1 µg/µL BSA, 0.0001% Tween-20, and 0. 0001% IGEPAL CA-630. While not limiting the technology to any particular FEN endonuclease, in preferred embodiments, cleavage reactions comprise a thermostable FEN-1 endonuclease selected from CLEAVASE 2.0 nuclease (Hologic, Inc.), *Archaeoglobus fulgidus* (Afu) FEN-1, *Pyrococcus furiosus* (Pfu) FEN-1, and *Archaeoglobus veneficus* (Ave) FEN-1 (see, *e.g.,* WO 02/070755).

In some embodiments, the composition comprises at least 5 unique flap oligonucleotides. In some embodiments, the composition comprises at least 10 unique flap oligonucleotides. In

some embodiments, the composition comprises at least 20 unique flap oligonucleotides. In some embodiments, the composition comprises at least 30 unique flap oligonucleotides. In some embodiments, the composition comprises at least 40 unique flap oligonucleotides. In some embodiments, the composition comprises at least 45 unique flap oligonucleotides, In some embodiments, the number of unique flap oligonucleotides corresponds to the number of target nucleic acids to be detected in the sample. In some embodiments, the composition contains multiple copies of a given flap oligonucleotide to ensure binding to the target nucleic acid, if present in the sample. For example, in some embodiments the composition comprises at least 5 unique flap oligonucleotides (*e.g.,* at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, or at least 45 unique flap oligonucleotides) and contains multiple copies of each unique flap oligonucleotide.

In some embodiments, the composition further comprises a flap endonuclease. In some embodiments, the flap endonuclease is a FEN-1 endonuclease, preferably a strict FEN-1 endonuclease.

In some embodiments, the composition further comprises ligatable molecules. In some embodiments, the ligatable molecules are also referred to herein as a "vector" or a "flap vector." In some embodiments, the ligatable molecules are circularizable molecules, *e.g.*, dumbbell probes or padlock probes, while in some embodiments, the ligatable molecules, form linear strands upon ligation, *e.g.*, when ligated to a cleaved flap.

In some embodiments, a vector or flap vector comprises a pair of molecules (a "vector pair"), wherein a first vector oligonucleotide comprises a first bridge-complementary region that is complementary to the 5′ end (*e.g.,* a 5′ end sequence) of a bridge oligonucleotide and a second vector oligonucleotide comprises a second bridge-complementary region that is complementary to the 3′ end (*e.g.,* a 3′ end sequence) of the bridge oligonucleotide, such that a third oligonucleotide, *e.g.*, a cleaved flap, hybridized to an inner segment of the bridge oligonucleotide is positioned between the two oligonucleotides of the vector pair, *e.g.*, as shown schematically in FIG. 14. FIG. 14 shows an embodiment in which Vector1 and Vector2 may be provided as separate vector molecules of a vector pair or may be connected by a backbone (not shown), as a circularizable vector molecule.

In some embodiments, the ligatable circularizable molecules are dumbbell probes. In some embodiments, each dumbbell probe comprises a bridge sequence that is complementary to a cleaved flap sequence. Accordingly, under suitable conditions a given dumbbell probe hybridizes to the cleaved flap sequence, generating ligatable nicks (as shown in FIG. 5B) which under suitable conditions are ligated to generate a dumbbell probe comprising a closed loop-stem-loop structure that can subsequently be amplified and/or detected, for instance, by PCR, RCA, RCT, sequencing, *etc.*

In some embodiments, the ligatable circularizable molecules are padlock probes. In some embodiments, each padlock probe comprises a first target region that is complementary to the 5′ end (*e.g.,* a 5′ end sequence) of a bridge oligonucleotide and a second target region that is complementary to the 3′ end (*e.g.,* a 3′ end sequence) of a bridge oligonucleotide. Accordingly, under suitable conditions a given padlock probe hybridizes to a bridge oligonucleotide with a single stranded segment (*i.e.,* the middle segment) remaining between the 3′ end and the 5′ end of the probe, which under suitable conditions hybridizes to a flap sequence or a cleaved flap sequence.

In some embodiments, the composition further comprises a plurality of bridge oligonucleotides, *e.g.*, bridge oligonucleotides to align cleaved flaps with one or more ligatable molecules (*e.g.,* a linear vector oligonucleotide or a vector pair, or a circularizable molecule such as a padlock probe or a dumbbell probe). In some embodiments, each bridge oligonucleotide comprises an inner segment that is complementary to a flap sequence. For example, in some embodiments each bridge oligonucleotide comprises an inner segment of 10-20 bases in length that is complementary to a flap sequence. In some embodiments, each bridge oligonucleotide comprises an inner segment that is complementary to a cleaved flap sequence. For example, in some embodiments each bridge oligonucleotide comprises an inner segment of, *e.g.,* 10-20 bases in length, that is complementary to a cleaved flap sequence, preferably to the full length of the cleaved flap sequence. In some embodiments, each bridge oligonucleotide additionally comprises a 5′ end sequence and a 3′ end sequence that are not complementary to the flap sequence or the cleaved flap sequence, and thus do not bind to the flap sequence or the cleaved flap sequence. In some embodiments, the 5′ end sequence and the 3′ end sequence each comprise at least three bases. In some embodiments, the 5′ end sequence and the 3′ end sequence each

comprise 3-30 bases. In some embodiments, the 5′ end sequence and the 3′ end sequence each comprise 10-25 bases. In some embodiments, the 5′ end sequence and the 3′ end sequence each comprise about 18 bases. In some embodiments, the 3′ and 5′ end sequences comprise the same number of nucleotides and in some embodiments, they comprise different numbers of nucleotides.

In some embodiments, the cleaved flaps act as circularizable molecules. In some embodiments, a bridge oligonucleotide is used to align the 3′ and 5′ ends of the cleaved flap for ligation. For example, in some embodiments, each cleaved flap comprises a first region that is complementary to the 5′ end (*e.g.,* a 5′ end sequence) of a bridge oligonucleotide and a second region that is complementary to the 3′ end (*e.g.,* a 3′ end sequence) of the bridge oligonucleotide. Accordingly, under suitable conditions, a given cleaved flap hybridizes to a bridge oligonucleotide at its 5′ end sequence and a 3′ end sequence, *e.g.*, preferably to form a ligatable nick. In some embodiments, the 5′ end sequence and the 3′ end sequence each comprise at least three bases. In some embodiments, the 5′ end sequence and the 3′ end sequence each comprise 3-30 bases. In some embodiments, the 5′ end sequence and the 3′ end sequence each comprise 10-25 bases. In some embodiments, the 5′ end sequence and the 3′ end sequence each comprise about 18 bases. In some embodiments, the 3′ and 5′ end sequences comprise the same number of nucleotides and in some embodiments, they comprise different numbers of nucleotides. In some embodiments, the cleaved flaps are ligatable in a non-circularized manner, *e.g.*, end-to-end to form a linear strand containing two or more cleaved flaps in a continuous strand.

In some embodiments, each ligatable molecule (*e.g.,* linear vector or vector pair, or circularizable molecule, such as padlock probe, dumbbell probe, or cleaved flap) further comprises a primer binding site, *e.g.*, for amplification. For example, in some embodiments each ligtable molecule comprises a primer binding site, *e.g.*, PCR, RCA, RCT, sequencing, *etc*. The sequence primer binding site may be any suitable nucleotide sequence that serves as a hybridization site for a primer for initiating a sequencing reaction. In some embodiments, each ligatable molecule (*e.g.,* linear vector or vector pair, or circularizable molecule, such as padlock probe, dumbbell probe, or cleaved flap) comprises a unique sequencing barcode (*e.g.,* a unique molecular identifier, UMI) that can be determined by sequencing to assist in the determination of which specific ligatable molecule of a plurality of ligatable molecules binds and is ligated to a

given cleaved flap sequence, or which specific flap oligonucleotides were cleaved in the cleavage reaction. In some embodiments, the UMI comprises 6-50 nucleotides. In some embodiments, the UMI comprises 10-40 nucleotides, 10-30 nucleotides, or 10-20 nucleotides. For example, in some embodiments the UMI comprises 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides. A nucleic acid molecule may comprise one UMI, or multiple different UMI sequences. In some embodiments, each ligatable molecule or flap portion, *e.g.*, of a flap probe, comprises one or more sequencing adapters (*e.g.,* binding sites for sequencing primers).

In some embodiments, each ligatable vector molecule or each ligatable vector pair further comprises an index sequence. As used herein, the terms "index sequence" and "indexing sequence" are used interchangeably and refer to a unique sequence of nucleotides useful for sample barcoding. For example, an index sequence is useful for identifying which population of molecules (*e.g.*, linear or circular ligated molecules formed in a ligation assay or replicated, *e.g.*, in an amplification assay) are associated with which specific sample or patient. Each molecule in a given population of molecules comprises the same index sequence, and each population of molecules comprises a different index sequence from each other population of molecules. For example, molecules in population "A" comprise index sequence "A," molecules in population "B" comprises index sequence "B," *etc.*

In some embodiments, the composition comprises a plurality of ligatable vector molecules or vector pairs, wherein each ligatable molecule vector pair contains the same number of nucleotides (*e.g.,* is the same size). For example, in some embodiments the composition comprises a plurality of ligated molecules, wherein each ligated molecule comprises the same number of nucleotides (*e.g.* is the same size) and comprises the same components with the exception of the UMI, which is a randomized sequence unique to each ligatable molecule thereby enabling specific identification of a given ligated molecule (*e.g.* after ligation of the cleaved flap sequence) during sequencing. In some embodiments, the size (*e.g.,* number of bases) of each UMI for each probe is the same (*e.g.,* 6-20 bases), but the sequence of the UMI differs for each ligatable molecule. In some embodiments, the composition comprises a plurality of ligatable molecules, wherein the size of some ligatable molecules differs from the size of other ligatable molecules in the composition. For example, in some embodiments, the composition comprises a first subset of ligatable molecules comprising a first number of total bases, a second

subset of ligatable molecules comprising a second number of total bases, a third subset of ligatable molecules comprising a third number of total bases, *etc.* Any suitable number of subsets of ligatable molecules can be used and any suitable number of bases for the different subsets of ligatable molecules can be used. In some embodiments, the number of bases for the first subset of ligatable molecules is N, the number of bases for the second subset of ligatable molecules is N-1, the number of bases for the third subset of ligatable molecules is N-2, the number of bases for the fourth subset of ligatable molecules is N-3, and so on. In some embodiments, a plurality of vector oligonucleotides or vector pairs (*e.g.*, usable to form a plurality of linear ligation products) may be used in place of the plurality of ligatable molecules to provide the elements described above.

In some embodiments, the composition comprises a plurality of circular probes, *e.g.*, circular templates as illustrated in FIG. 4, wherein each circular probe contains the same number of nucleotides (*e.g.,* is the same size). For example, in some embodiments the composition comprises a plurality of circular probes, wherein each circular probe comprises the same number of nucleotides (*e.g.* is the same size) and comprises the same components with the exception of the UMI, which is a randomized sequence unique to each circular probe thereby enabling specific identification of a given circular probe (*e.g.* after extension of a cleaved flap sequence acting as a primer) during sequencing. In some embodiments, the size (*e.g.,* number of bases) of each UMI for each probe is the same (*e.g.,* 6-20 bases), but the sequence of the UMI differs for each circular probe. In some embodiments, the composition comprises a plurality of circular probes, wherein the size of some circular probes differs from the size of other circular probes in the composition. For example, in some embodiments, the composition comprises a first subset of circular probes comprising a first number of total bases, a second subset of circular probes comprising a second number of total bases, a third subset of circular probes comprising a third number of total bases, *etc.* Any suitable number of subsets of circular probes can be used and any suitable number of bases for the different subsets of circular probes can be used. In some embodiments, the number of bases for the first subset of circular probes is N, the number of bases for the second subset of circular probes is N-1, the number of bases for the third subset of circular probes is N-2, the number of bases for the fourth subset of circular probes is N-3, and so on.

In some embodiments, a ligatable molecule (*e.g.*, a circularizable molecule or linear vector molecule or vector pair) comprises a 3′ terminal ribonucleotide. In some embodiments, a 3′ terminal ribonucleotide present on a ligatable molecule prevents extension of the molecule by a polymerase, such as residual polymerase remaining following a PCR-flap assay (*e.g.,* an LQAS reaction, as described above).

In some embodiments, the composition further comprises a ligase, preferably a high-fidelity ligase. In some embodiments, the composition further comprises a source of phosphate groups to be added to the cleaved flap sequences to facilitate ligation. In some embodiments, the flap sequences are phosphorylated prior to cleavage. In some embodiments, the composition further comprises Mg^{2+}. In some embodiments, the composition further comprises one or more primer pairs for amplification of target nucleic acid. In some embodiments, the composition further comprises nucleic acid extracted from a biological sample obtained from a subject.

In some aspects, provided herein are multiplexed methods for detecting a plurality of target nucleic acids in a sample. In some embodiments, methods for multiplexed detection of a plurality of target nucleic acids in a sample comprise contacting the sample with a composition comprising a plurality of flap oligonucleotides as described herein.

In some embodiments, methods for multiplexed detection of a plurality of targets comprise contacting a sample containing or suspected of containing target nucleic acids with a composition comprising a plurality of flap oligonucleotides under suitable conditions (*e.g.,* in a buffer suitable for sequence-specific hybridization of DNA, *e.g.,* in the presence of mono-or divalent cations), such that the target-specific region hybridizes to the target sequence. In some embodiments, hybridization of the target-specific region to the target nucleic acid occurs in the presence of an endonuclease, preferably in the presence of a strict thermostable FEN-1 enzyme, such that cleavage of the flap sequence occurs when hybridization of the target-specific region to the target nucleic acid forms an invasive cleavage structure.

An aspect of the technology is use of a flap endonuclease under conditions in which the enzyme exhibits maximum specificity for an invasive cleavage structure, *e.g.,* as illustrated in FIG. 1, as compared to, *e.g.,* a gapped structure (having a single-stranded gap between the upstream and downstream duplexes), Y-structure (having a 5′ flap but no overlap between the

upstream and downstream duplexes), pseudo-Y structure (having a 5′ flap and an un-duplexed template strand upstream of the downstream duplex), *etc.* Thus, in preferred embodiments, cleavage of hybridized padlock probes is done in a Mg^{++} flap assay buffer. As used herein, the term "Mg^{++} flap assay buffer" refers to a buffer solution for a flap endonuclease that includes Mg^{++} as the predominant or essentially only divalent cation in the buffer, such that cleavage reactions performed in the buffer, *e.g.,* cleavage of hybridized padlock probe that forms an invasive cleavage structure, exhibits characteristics of the presence of Mg^{++}, and does not exhibit characteristics associated with the presence of other divalent cations (*e.g.,* alternative cleavage activities of flap endonucleases associated with use of Mn^{++} with or in place of Mg^{++}), as described, *e.g.,* by Kaiser, *et al*., *supra*.

In some embodiments the flap assay buffer has very low or no KCl and comprises elevated Mg^{++} as compared to standard PCR buffer, (*e.g.,* a flap assay buffer of the technology comprises >6 mM, preferably > 7mM, more preferably 7.5mM Mg^{++}, while PCR buffers typically comprise about 1.5 to 2.5 mM Mg^{++}). In preferred embodiments, cleavage is conducted in a flap assay buffer comprising 7.5 mM $MgCl_2$, 10 mM MOPS, 0.3 mM Tris-HCl, pH 8.0, 0.8 mM KCl, 0.1 µg/µL BSA, 0.0001% Tween-20, and 0. 0001% IGEPAL CA-630. While not limiting the technology to any particular FEN endonuclease, in preferred embodiments, cleavage reactions comprise a thermostable FEN-1 endonuclease selected from Cleavase 2.0 nuclease (Hologic, Inc.), *Archaeoglobus fulgidus* (Afu) FEN-1, *Pyrococcus furiosus* (Pfu) FEN-1, and *Archaeoglobus veneficus* (Ave) FEN-1 (see, *e.g.,* WO 02/070755).

In some embodiments, the cleaved flap sequence is ligated to a circularizable molecule, thereby forming a closed circular structure which can subsequently be amplified and/or detected. In some embodiments, the circularizable molecule is a padlock probe. In some embodiments, the circularizable molecule is a dumbbell probe. In some embodiments, the 3′ and 5′ ends of a cleaved flap are ligated together, thereby forming a closed circular structure that can subsequently be amplified and/or detected.

In some embodiments, the cleaved flap sequence is ligated to a linear vector molecule, preferably a pair of linear vector molecules, thereby forming a linear ligated molecule comprising the vector and flap sequences, which can subsequently be amplified and/or detected.

In some embodiments, the 3′ end of the flap is ligated to a vector molecule, and in some embodiments, the 3′ and 5′ ends of a cleaved flap are ligated to different vector molecules in a vector pair.

In some embodiments, the cleaved flap sequence hybridizes to a bridge oligonucleotide. The bridge oligonucleotide may be present in the composition comprising the plurality of flap oligonucleotides, as described above, or may be added to the sample following contacting the sample with the composition. In some embodiments, the cleaved flap sequence hybridizes to an inner segment of the bridge oligonucleotide, as described above, whereas the 3′ end and the 5′ end of the bridge oligonucleotide do not hybridize to the cleaved flap sequence. In some embodiments, the uncleaved flap sequence (*e.g.,* the flap sequence of the uncleaved flap oligonucleotide) is able to hybridize to the bridge oligonucleotide, even in the absence of cleavage by the endonuclease.

In some embodiments, ligatable vector molecules are present in the composition or are added to the sample following contacting the sample with the composition. In some embodiments, ligatable vector molecules (*e.g.,* padlock probes, dumbbell probes) generate a closed circular structure containing the cleaved flap sequence, while in some embodiments, the ligatable vector molecules generate a linear ligated product containing the cleaved flap sequence.

In some embodiments, the circularizable molecules are padlock probes, and each padlock probe comprises a first target region that is complementary to the 5′ end (*e.g.,* a 5′ end sequence) of a bridge oligonucleotide and a second target region that is complementary to the 3′ end (*e.g.,* a 3′ end sequence) of the bridge oligonucleotide. Accordingly, under suitable conditions a given padlock probe hybridizes to the bridge oligonucleotide whereas, as described above, the inner segment of the padlock probe hybridizes to the cleaved flap sequence. In some embodiments, the circularizable molecules are dumbbell probes, and each dumbbell probe comprises a bridge sequence that is complementary to the cleaved flap sequence such that the cleaved flap sequence hybridizes to the bridge sequence of a dumbbell probe.

In some embodiments, the ligatable molecules comprise a pair of vector oligonucleotides that are similar to the ends of the padlock and dumbbell probes described above. For example, in some embodiments the ligatable molecules comprise a first vector oligonucleotide comprising a

first target region that is complementary to the 5′ end (*e.g.,* a 5′ end sequence) of a bridge oligonucleotide and a second vector oligonucleotide comprises a second target region that is complementary to the 3′ end (*e.g.,* a 3′ end sequence) of the bridge oligonucleotide. Accordingly, under suitable conditions a given vector pair hybridizes to each end of the bridge oligonucleotide, and the inner segment of the bridge oligonucleotide hybridizes to the cleaved flap sequence.

In some embodiments, the cleaved flap sequence may hybridize to the circularizable molecule and serve as a primer, *e.g.*, to replicate a circular template (as shown in FIG. 3). In some embodiments, the circularizable molecule may already be circularized prior to hybridization of the cleaved flap sequence. In some embodiments, the cleaved flap sequence may hybridize to a linear nucleic acid strand, *e.g.*, to act as a primer that the linear nucleic acid strand as template for replication.

Ligation of ligatable molecules

In some embodiments, the cleaved flap sequence is ligated to a flap vector, *e.g.*, a circularizable molecule or pair of vector oligonucleotides, thereby forming a closed circular or linear ligated structure, respectively. Ligation of a 5′ end of nucleic acid to a 3′ end of nucleic acid can occur when the two ends are base paired to adjacent nucleotides of a complementary sequence, and wherein the 5′ terminal nucleotide has a 5′ phosphate and the 3′ terminal nucleotide has a 3′ hydroxyl group. Base pairing of the respective end nucleotides to the adjacent nucleotides in a template strand forms a nucleic acid duplex containing a nick. Ligation of the two ends at the nick can be catalyzed by DNA ligase. Providing conditions for ligation will therefore usually comprise providing a DNA ligase enzyme and reaction conditions under which the DNA ligase ligates the two ends to form a continuous nucleic acid strand, closing the nick. Preferably, a high-fidelity ligase is used under conditions that maximize mismatch discrimination. For example, an amount of HiFi Taq DNA Ligase (New England Biolabs) is used, preferably in a buffer of 20 mM Tris-HCl; 150 mM KCl; 10 mM $MgCl_2$; 10 mM DTT; 1 mM NAD; 0.1% Triton® X-100; (pH 8.5 @ 25°C) and is incubated at between 37-75°C for 10-60 min, or by thermal cycling at 95°C for 30 s to 1 min, followed by annealing/ligation for 1 – 5 min at a ligation temperature selected for a particular probe used. A number of other ligase

enzymes are commercially available, including Ampligase (Epicentre), for which suitable conditions are to add 1 U enzyme and incubate at 55° C. for 1 hour in ligase buffer. In some embodiments, ligation occurs in 10 mM Tris-acetate pH 7.5, 10 mM magnesium acetate, 50 mM NaCl, 1 mM ATP, 1 μg/mL BSA and 0.2 U/μL T4 DNA ligase (Amersham Pharmacia Biotech, Uppsala, Sweden) at 37°C for 30 min in the presence of the target DNA (*e.g.,* the cleaved flap sequence). See, *e.g.,* Nilsson, *et al*., Nucl. Acids Res. 30(14):e66 (2002) and Lohman, G. *et al*. (2016). Nucleic Acids Res. 44(2):e14-e14 DOI: 0.1093/nar/gkv898, each of which is incorporated herein by reference in its entirety.

In some embodiments, a cleaved flap sequence is ligated to the ligatable molecule(s) (*e.g.,* padlock probe, dumbbell probe, linear vector oligonucleotide or vector pair) in the same buffer in which flap endonuclease cleavage occurs. In certain embodiments, the sample is contacted with a composition comprising flap oligonucleotides, a FEN endonuclease, bridge oligonucleotides, padlock probes, or linear vector oligonucleotide or vector pair, and a ligase enzyme. In certain embodiments, the sample is contacted with a composition comprising flap oligonucleotides, a FEN endonuclease, dumbbell probes, and a ligase enzyme.

In some embodiments, a cleaved padlock probe or vector oligonucleotide or vector pair is ligated in the same buffer in which FEN cleavage occurs, while in some embodiments, the cleavage reaction mixture is desalted or diluted prior to ligation, *e.g.,* to provide buffer conditions more suitable for a selected ligase. In some embodiments, reaction conditions for cleavage are selected for compatibility with a selected ligase, whereby the cleaved probes may be used in a ligation reaction without dilution or desalting.

For example, in some embodiments, cleavage and ligation are performed in a buffer comprising $MgCl_2$, and ATP (*e.g.,* 10 mM MOPS, pH 7.5, 2 mM $MgCl_2$, 20 μg/ml tRNA, 1 mM ATP; see Kaiser *et al., supra*).

In some embodiments, a reaction mixture comprises target nucleic acids, vector probes (*e.g.*, padlock probes, dumbbell probes, linear vector oligonucleotides, or vector pairs, FEN endonuclease, and a ligase enzyme. It has been shown that HiFi *Taq* DNA Ligase can be used in alternative buffers, *e.g., Taq* DNA Ligase buffer with reduced activity and fidelity. HiFi *Taq* DNA Ligase can also be used in a variety of polymerase buffers when supplemented

with 1 mM NAD, with reduced activity and modestly reduced fidelity:

NEB Buffer	HiFi Taq DNA Ligase Activity (%)	Effect on Fidelity
Hifi *Taq* DNA Ligase Buffer	100	-
Taq DNA Ligase Buffer	38	↓↓↓
phi29 Reaction Buffer[1]	54	↓↓
Q5® Reaction Buffer[1]	73	↓
Standard *Taq* Reaction Buffer[1]	90	↓
ThermoPol Buffer®[1]	215	↓↓

(see www<dot>neb<dot>com/faqs/2016/03/18/can-hifi-taq-dna-ligase-be-used-in-other-buffers)

[1] Supplemented with 1 mM NAD

- 1X HiFi Taq DNA Ligase buffer:

  20 mM Tris-HCl; 150 mM KCl; 10 mM $MgCl_2$; 10 mM DTT; 1 mM NAD; 0.1% Triton® X-100; (pH 8.5 @ 25°C)

- *Phi29* Reaction buffer:

  50 mM Tris-HCI pH 7.5, 10 mM MgCl2, 10 mM $(NH_4)SO_4$, 4 mM dithiothreitol (pH 7.5 @ 25°C)

- Standard Taq reaction buffer conditions:

  10 mM Tris-HCl; 50 mM KCl; and1.5 mM $MgCl_2$ (pH 8.3 @ 25°C).

- Exemplary flap assay reaction buffer:

7.5 mM $MgCl_2$, 10 mM MOPS, 0.3 mM Tris-HCl, pH 8.0, 0.8 mM KCl, 0.1 µg/µL BSA, 0.0001% Tween-20, and 0. 0001% IGEPAL CA-630

It is contemplated that the similarities of these buffers are indicative that this and other ligases are compatible with conditions suitable for DNA polymerase and FEN-1 endonuclease activities. For example, as noted above, Kaiser performed cleavage and ligation in a buffer comprising 10 mM MOPS, pH 7.5, 2 mM $MgCl_2$, 20 μg/ml tRNA, and 1 mM ATP (see Kaiser *et al., supra*).

In some embodiments, the cleaved flap sequence is ligated to the circularizable molecule, linear vector oligonucleotide, or vector pair in a separate reaction, which occurs following flap endonuclease cleavage. For example, in some embodiments an invasive cleavage assay is conducted by contacting a sample with a composition comprising a flap oligonucleotide and a flap endonuclease under conditions such that an invasive cleavage structure comprising the flap oligonucleotide and a target nucleic acid forms, if the target nucleic acid is present in the sample and the flap sequence is cleaved from the flap oligonucleotide. In some embodiments (*e.g.*, as shown in FIG. 1) the composition comprises an invasive oligonucleotide which serves as the upstream oligonucleotide in the invasive cleavage structure. In some embodiments, the invasive oligonucleotide is designed to be complementary to a portion of the target nucleic acid strand which is not bound to the target-specific region of the flap oligonucleotide. In some embodiments (*e.g.*, as shown in FIG. 2), the flap oligonucleotide comprises a both a flap portion and an invasive portion, wherein the flap portion and invasive portion of the flap oligonucleotide each bind to different but adjacent regions on the target nucleic acid, thereby forming the upstream and downstream duplexes in the invasive cleavage structure.

Ligation of circularizable cleaved flaps

In some embodiments, a cleaved flap is treated under suitable conditions such that the 5′ end and the 3′ end of the cleaved flap strand hybridize a splint oligonucleotide thereby aligning the ends of the cleaved flap to form a ligatable nick, *e.g.,* as shown schematically in FIG. 6B, left panel, wherein ligation of the nick forms a circularized flap. Providing conditions for ligation will therefore be the same or similar to ligation of circularizable molecules, described above. For example, ligation will usually comprise providing a DNA ligase enzyme and reaction conditions under which the DNA ligase ligates the two ends to form a continuous nucleic acid strand,

closing the nick,. In preferred embodiments, a high-fidelity ligase is used under conditions that maximize mismatch discrimination. For example, an amount of HiFi Taq DNA Ligase (New England Biolabs) is used, preferably in a buffer of 20 mM Tris-HCl; 150 mM KCl; 10 mM $MgCl_2$; 10 mM DTT; 1 mM NAD; 0.1% Triton® X-100; (pH 8.5 @ 25°C) and is incubated at between 37-75°C for 10-60 min, or by thermal cycling at 95°C for 30 s to 1 min, followed by annealing/ligation for 1 – 5 min at a ligation temperature selected for a particular probe used. A number of other ligase enzymes are commercially available, including Ampligase (Epicentre), for which suitable conditions are to add 1 U enzyme and incubate at 55° C. for 1 hour in ligase buffer. In some embodiments, ligation occurs in 10 mM Tris-acetate pH 7.5, 10 mM magnesium acetate, 50 mM NaCl, 1 mM ATP, 1 µg/mL BSA and 0.2 U/µL T4 DNA ligase (Amersham Pharmacia Biotech, Uppsala, Sweden) at 37°C for 30 min in the presence of the target DNA (*e.g.,* the cleaved flap sequence). See, *e.g.,* Nilsson, *et al*., Nucl. Acids Res. 30(14):e66 (2002) and Lohman, G. *et al*. (2016). Nucleic Acids Res. 44(2):e14-e14 DOI: 0.1093/nar/gkv898, each of which is incorporated herein by reference in its entirety. In another example, a cleaved flap may be combined in a reaction comprising a splint oligonucleotide and ligated in a process comprising combining the cleaved flap and split oligonucleotide in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$. After denaturation for 5 min at 75°C, the solution is allowed to slowly reach room temperature. ATP, DTT and BSA are added to final concentrations of 100 µM, 10 mM and 25 µg/ml, respectively. The reaction is initiated by adding T4 DNA ligase to a final concentration of 800 U/ml. The reaction is incubated, *e.g.*, for 12 h at room temperature, after which the ligase is heat-inactivated. (see, *e.g.*, JS Hartig, *et al*., Nucleic Acids Res. 2004; 32(19): e152) In embodiments in which hybridization of the cleaved flap with a splint oligonucleotide results in another flap structure, the complex may be treated with a flap endonuclease, *e.g.*, a FEN-1 endonuclease, to remove a 5′ flap and leave a ligatable nick.

In some embodiments, a ligase that is competent to ligate single-stranded ends, *e.g.*, T4 RNA ligase, CIRCLIGASE ™ ssDNA ligase, or CIRCLIGASE II™ ssDNA ligase, is used to ligate the 5′ and 3′ ends of a cleaved flap to each other to form a circularized flap, as shown schematically in FIG. 6B, right panel. By way of example and not by limitation, a cleaved flap may be combined in a ligation reaction mixture containing 50 mM HEPES, pH 8.0, 66 mM KOAc, 0.5 mM DTT, 1 M betaine, and 30 U CIRCLIGASE II™ (Epicentre Biotechnologies,

Madison, WI) at 60° C. for 2 hours. The ligase is subsequently heat-inactivated, *e.g.*, by incubating the reaction mixture at 80° C. for 10 minutes. See, *e.g.*, US Pat. No. 9938568 to RC Heller, *et al*., which is incorporated herein by reference in its entirety, for all purposes.

In some embodiments, cleavage of a flap oligonucleotide (if it has occurred) is detected in a subsequent reaction. Accordingly, in some embodiments, following contacting the sample with the composition comprising a flap oligonucleotide and a flap endonuclease under conditions such that an invasive cleavage structure comprising the flap oligonucleotide and a target nucleic acid forms (*e.g.,* if the target nucleic acid is present in the sample), cleavage of the flap sequence is detected by a method comprising use of one or more of a padlock probes (including but not limited to, *e.g.,* a sequence library vector), bridge oligonucleotides, splint oligonucleotides, a ligase enzyme, a DNA polymerase, and one or more primers. For example, in some embodiments, a cleaved flap is treated under suitable conditions such that the cleaved flap strand hybridizes to an inner segment of a bridge oligonucleotide and the 5′ end and the 3′ ends of the bridge oligonucleotide hybridize to the first end and a second target end of the padlock probe, respectively, thereby aligning the cleaved flap and the ends of the padlock probe to form 2 ligatable nicks, *e.g.,* as shown schematically in FIG. 5A. A subsequent ligation forms a closed circular strand of nucleic acid comprising the padlock probe sequence and the cleaved flap sequence.

In some embodiments, following contacting the sample with the composition comprising a flap oligonucleotide and a flap endonuclease under conditions such that an invasive cleavage structure comprising the flap oligonucleotide and a target nucleic acid forms (if the target nucleic acid is present in the sample), cleavage of the flap is detected using a second composition containing dumbbell probes and ligase enzymes, and performing a reaction under suitable conditions such that the cleaved flap sequence hybridizes to the bridge sequence of the dumbbell probe. In certain embodiments, the method comprises use of one or more dumbbell probes comprising bridge sequences (including but not limited to, *e.g.,* a sequence library vector), a ligase enzyme, a DNA polymerase, and one or more primers. For example, in some embodiments, a cleaved flap is treated under suitable conditions such that the cleaved flap strand hybridizes to an inner segment of a bridge sequence of a dumbbell probe, thereby aligning the cleaved flap and the ends of the dumbbell probe to form 2 ligatable nicks, *e.g.,* as shown

schematically in FIG. 5B. A subsequent ligation forms a closed circular strand of nucleic acid (*e.g.,* a closed loop-stem-loop structure) comprising the dumbbell probe sequence and the cleaved flap sequence.

In some embodiments, following contacting the sample with the composition comprising a flap oligonucleotide and a flap endonuclease under conditions such that an invasive cleavage structure comprising the flap oligonucleotide and a target nucleic acid forms (if the target nucleic acid is present in the sample), the cleaved flap is treated under conditions wherein the ends of the cleaved flap are ligated together to form a circularized flap sequence, as shown schematically in FIGS. 6B and 6C.

Enhancing ligation of cleaved flaps

Carryover contamination from residual polymerase (*e.g.,* GoTaq polymerase) from a PCR-flap assay can reduce ligation sensitivity and overall analytical sensitivity of the method, *e.g.,* by inhibiting hybridization of the cleaved flap sequence to the bridge oligonucleotide. Thus, in one exemplary method, following formation of cleaved flap sequences by an invasive cleavage assay in the presence of polymerase (*e.g.* a PCR-flap assay such as an LQAS assay), the reaction mixture is treated with a phosphatase, *e.g.,* Calf Intestinal Alkaline Phosphatase (CIP) or Shrimp alkaline phosphatase (rSIP) to dephosphorylate dNTPs in the reaction mixture, thereby inhibiting polymerase activity from residual polymerase used in the PCR-flap assay. See, *e.g.,* New England Biolabs Application note "Enzymatic PCR Cleanup using Exonuclease I and Shrimp Alkaline Phosphatase" (2019), for use of rSAP, NEB Cat. #M0371. (*e.g.,* 1µL of rSAP combined with 5 µL of PCR product, incubated at 37°C for 15 min., heated to 80°C for 15 min. to deactivate prior to downstream processing of the PCR products).

Polymerase activity may also be limited by using a low ligation temperature, *e.g.,* below the temperature at which the polymerase is active, by degrading the polymerase with an appropriate proteinase (*e.g.,* Proteinase K) after the PCR-flap assay, or by heat killing the polymerase (*e.g.,* at 99°C for 10 min.)

Treatment with CIP typically also removes 5′ phosphates from oligonucleotides, *e.g.,* from the 5′ end of cleaved flaps, if present. In preferred embodiments, the cleaved flaps are treated with a polynucleotide kinase (PNK), *e.g.,* T4 polynucleotide kinase (T4 PNK). In some embodiments, PNK is used under suitable conditions to phosphorylate the 5′ end of the cleaved flap and/or the 5′ end of the ligatable molecule (*e.g.*, the circularizable molecule or a 5′ ligatable end of a linear vector oligonucleotide), to enable ligation of the cleaved flap to the circularizable molecule or vector oligonucleotide.

The cleaved flap and a circularizable molecule may be hybridized to a bridging or splint oligonucleotide or sequence, *e.g.,* as shown schematically in Figs. 5A and 5B, or the cleaved flap and vector pair may be hybridized to bridging or splint oligonucleotide, *e.g.*, as shown schematically in FIG. 14, forming ligatable nicks. The phosphorylated cleaved flap and the ligatable molecules in a hybridized structure are then ligated using a ligase enzyme, *e.g.,* HiFi Taq DNA ligase.

In embodiments in which the ligatable molecule is circularizable, a the ligation forms a covalently closed circular nucleic acid. Following ligation, residual non-circular molecules, *e.g.,* un-circularized vector, residual oligonucleotides, and other nucleic acid material, are removed by digestion with an appropriate exonuclease, *e.g.*, Lambda Exonuclease, Exonuclease III, Exonuclease I, or Exonuclease VIII, such that the closed circular structures are suitable for downstream applications, *e.g.,* amplification and sequencing.

Ligation of cleaved flaps using RNA bridging sequences

In some embodiments, activity of residual DNA polymerase, *e.g.*, from a PCR-flap assay reaction, is limited by using bridging or splint strands comprising RNA. For example, in some embodiments a cleaved flap is ligated to a ligatable molecule, *e.g.*, a circularizable flap vector or linear vector pair, using an RNA bridge oligonucleotide, or a chimeric bridge oligonucleotide in which at least a portion of the oligonucleotide strand comprises RNA, *e.g.*, as shown schematically in Fig. 14.

As discussed above, in certain embodiments for making a ligatable structure, the 3′ and 5′ ends of a flap vector are aligned by the bridge oligonucleotide to be adjacent to the 5′ and 3′ ends of the cleaved flap, to form nicks. Using a ligase suitable for ligating a DNA nick on an RNA or RNA-analog bridge strand, *e.g.*, SPLINTR ligase (also known as PBCV-1 DNA ligase or *Chlorella* virus DNA ligase, New England BioLabs; see also Krzywkowski, T. and M. Nilsson, *Nucleic Acids Research,* 2017, Vol. 45, No. 18:e161, and Shin, M et al., Nucleic Acids Therapeutics v32 (1):66-72 (2022); each of which is incorporated herein by reference in its entirety, for all purposes), the ends of the cleaved flap are ligated to the flap vector to form a circularized molecule.

The bridge oligonucleotide may be completely RNA, or may be RNA in selected portions, *e.g.*, where the 3′ end of the flap vector hybridizes. In some embodiments, the RNA portions of the bridge oligonucleotide comprise or are composed of ribonucleotide analogs, *e.g.*, 2′ modified ribonucleotides, such as 2′-O-methyl or 2′-O-methoxyethyl nucleotides. 2′-O-methyl oligonucleotides have the general structure of RNA oligonucleotides but are more stable than RNA oligonucleotides, *e.g.*, they are more resistant to nucleases.

In some embodiments, the 5′ end of the flap vector is configured to be unligatable unless activated, *e.g.*, by cleavage to remove the 5′ terminus (termed a "5′ activatable flap vector"). For example, as illustrated in Fig. 14, in some embodiments the 5′ terminus of the flap vector is not phosphorylated, and/or is engineered to have an unpaired arm or flap, *e.g.*, a 2-nucleotide 5′ arm that is not complementary to the bridge oligonucleotide. Once a cleaved flap having the correct 3′ terminus is hybridized to the bridge oligonucleotide along with the flap vector, a flap endonuclease cleaves the 5′ arm from the flap vector, leaving a 5′ phosphate that is ligatable to the 3′ end of the cleaved flap. In preferred embodiments, the flap endonuclease comprises a FEN-1 endonuclease carried over from a PCR-flap assay step. Use of a chimeric bridge oligonucleotide, *e.g.*, as illustrated in Fig. 14, provides a suitable DNA template strand for use with a FEN-1 endonuclease that may exhibit reduced activity on an RNA strand. As noted above, while a bacterial flap endonuclease, *e.g.*, a 5′ nuclease of a DNA polymerase, may cleave a DNA probe in an invasive cleavage structure formed on an RNA template strand, a strict FEN-1 endonuclease will not. See, *e.g.*, US Pat. No. 7,045,289 to Allawi, *et al*.

Enrichment of ligated products

A method may include enriching the ligation products (*e.g.,* enriching the ligated, closed circular structure comprising the circularizable molecule and a cleaved flap sequence or linear ligated products comprising a vector oligonucleotide or vector pair and a cleaved flap sequence) before detection. Products may be enriched by amplification and/or by solid phase chemistry. Circular nucleic acid products may be selectively enriched by treating the sample with exonuclease (*e.g.,* Lambda Exonuclease, Exonuclease III, Exonuclease I, or Exonuclease VIII) to digest linear nucleic acid products, *e.g.,* target nucleic acid fragments, unligated padlock probes, *etc.* In general, exonuclease degradation may be used to enrich for ligation products when the ligation products are protected from exonuclease degradation. Exonuclease should then be deactivated (*e.g.,* by heat) before any subsequent step involving polymerization, *e.g.,* before rolling circle amplification. See, *e.g.,* US Pat. No. 10,526,643, *supra*, and U.S. Patent No. 11,186,863, both of which are incorporated herein by reference in their entireties, for all purposes.

Exonuclease may be added to remove non-circularized oligonucleotides. Suitable conditions are incubation at 37°C for 1 hour in corresponding exonuclease buffer, followed by enzyme inactivation at 80°C for 20 minutes. Where capture/detect methods are used, ligation products may be enriched by capturing the products on a solid phase via the capture moiety. For example, as illustrated in example 1 of US Pat. No. 10,526,643, *supra*, a solution containing ligation products may be mixed with 10 mL M-280 streptavidin coated magnetic beads (Invitrogen) in Tris-HCI (pH 7.5), 3.5 mM EDTA and 0.07% Tween-20 in a final volume of 200 ml, and incubated at room temperature for 15 minutes. After incubation, the beads are collected using a magnet and supernatant is removed. See also Example 3 in U.S. Patent No. 11,186,863. Other ways of enriching for ligation products include specifically size-selecting ligation products.

As discussed above, use of an RNA or chimeric bridge oligonucleotide and a 5′ activatable flap vector at the ligation step may reduce or eliminate the advantages of using this enrichment step.

Amplification of ligated products

In some embodiments, the closed circular structures or linear ligated products are subsequently amplified and detected. A number of different nucleic acid assays may be used, *e.g.,* PCR (*e.g.,* across the ligated junction), ligase chain reaction, reporter probe hybridization, rolling circle amplification, rolling circle transcription, (see, *e.g.*, *e.g.,* by SL Daubendiek, *et al*. J Am Chem Soc. 1995;117:7818–7819, which is incorporated herein by reference in its entirety, for all purposes.) *etc.* Suitable amplification techniques include but are not limited to rolling circle amplification (see below; also see JS Hartig, *et al*., Nucleic Acids Res. 2004; 32(19): e152)), bridge PCR (Adessi C, *et al*., Nucleic Acids Res. 2000 Oct 15;28(20):E87), emulsion PCR (digital PCR in emulsions was described by Dressman *et al*., Proc Natl Acad Sci U S A. 2003 Jul 22;100(15):8817-22. Epub 2003 Jul 11) and digital PCR (Vogelstein & Kinzler, Proc Natl Acad Sci U S A. 1999 Aug 3;96(16):9236-41), each of which is incorporated herein by reference in its entirety, for all purposes. Clonal localized amplification in gels was described by Mitra & Church, Nucleic Acids Res. 1999 December 15; 27(24): e34, which is incorporated herein by reference in its entirety, for all purposes. An embodiment of the present method may comprise amplifying the ligation products and obtaining a cumulative signal which is a combination of individual signals from the amplified products. Preferably, ligation products are amplified across the ligation junction.

In certain preferred embodiments, the ligated products are amplified as a step in preparing a library, *e.g.*, a sequencing library. For example, in some embodiments, the amplicons are produced by amplifying a region of the ligated molecule mixture using NGS indexing primers that prime from adapter sequences in the circularizable or vector molecule-portion of the product mixture, as shown schematically in FIGS. 9A and 14. See also, Bohmann K, *et al*., "Strategies for sample labelling and library preparation in DNA metabarcoding studies" *Mol Ecol Resour.* 2022;22:1231–1246; and US Pat. No. 10,526,643B2, each of which is incorporated herein by reference in its entirety, for all purposes. In some embodiments, ligatable molecules, such as vectors used in NGS methods, comprise an indexing sequence, *e.g.*, on the circularizable molecule or vector, as shown schematically in FIG. 8, 9B, and 14. In some embodiments, the

ligatable molecule(s) comprising an indexing sequence may be used in addition to, in place of, or in combination with indexing sequences on amplification primers. In some embodiments, flap sequences used in flap assays or PCR-flap assays comprise indexing sequences, which may be used in addition to, in place of, or in combination with indexing sequences on amplification primers and/or on the circularizable molecule or vector. Use of an indexing sequence on the vector and/or flap sequence provides additional indexing capacity, *e.g.*, if used in addition to the indexing capacity based on amplification primer indexing, thus allowing higher level multiplexing of samples analyzed in, for example, a single sequencing run. Indexing sequences in flap sequences and/or in the circularizable molecules or vector oligonucleotides find application in, for example, subject-specific (*e.g.*, patient-specific) and/or sample-specific indexing of data. In some embodiments, multiple vectors comprising different vector indexes may be used, *e.g.*, 2, 3, 4, 5, or more different indexing vectors. In other embodiments the ligatable molecules comprising an indexing sequence are not used in combination with indexing sequences on amplification primers.

In preferred embodiments, amplification may comprise providing conditions for rolling circle replication of the closed circular structures and detecting the products of rolling circle replication. Rolling circle replication is described in, for example, US 5,854,033 (Lizardi); Fire & Xu, Proc Natl Acad Sci U S A. 1995 May 9;92(10):4641 -5, and M. Ali, *et al.* "Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine". *Chemical Society Reviews*. **43** (10): 3324–3341, each of which is incorporated herein by reference in its entirety, for all purposes.

Rolling circle replication is an amplification of a circular nucleic acid molecule using a hybridized primer that is extended using strand-displacing DNA polymerase, resulting in large DNA molecules containing tens to hundreds of tandem repeats complementary to the circular template. (See, *e.g.,* M. Ali, *et al. supra*; see also US Pat. No. 10,526,643B2, which is incorporated herein by reference in its entirety, for all purposes.) Polymerases typically used in RCA are *Phi*29 (Φ29), Bst, and Vent exo$^-$ DNA polymerases, with *Phi*29 DNA polymerase being preferred in view of its superior processivity and strand displacement ability. Additional amplification can be obtained using a cascade of strand displacement reactions. For example, rolling circle replication may be hyper-branched rolling circle replication, as described by

Lizardi *et al*., Nat Genet. 1998 Jul;19(3):225-32, which is incorporated herein by reference in its entirety, for all purposes.

In some embodiments, the ligation reaction mixture is desalted or diluted prior to detection, *e.g.,* to provide buffer conditions more suitable for a selected polymerase. In some embodiments, reaction conditions for cleavage and/or ligation are selected for compatibility with a selected polymerase, whereby the ligated material may be used in an amplification reaction without dilution or desalting.

By way of non-limiting example, in some embodiments, polymerization reactions are performed using a primer hybridized to the ligated probe in 50 mM Tris-HCI pH 7.5, 10 mM MgCl2, 20 mM $(NH_4)SO_4$, 10 mM dithiothreitol, 0.2 μg/mL BSA, 0.25 mM dNTPs, and 2 ng/mL *Phi*29 DNA polymerase at 37°C. In some embodiments, primers for RCA are immobilized on a support, *e.g.,* a bead or other surface. See, *e.g.,* U.S., Pat. No. 6,316,229 to Lizardi and US Pat. No. 10,526,643B2, which is incorporated herein by reference in its entirety, for all purposes.

In some embodiments, RCA reactions may be monitored in real time, during the extension reaction. For example, the RCA may be performed in the presence of 100 nM of a molecular beacon probe that is complementary to the concatemer product and 300 nM ROX dye. Molecular beacon probes typically comprise a FRET system comprising a fluorophore that is quenched in the unhybridized probe and that is unquenched when the probe hybridizes to a target sequence. The reactions are followed in real time by measuring fluorescence, *e.g.,* in an ABI 7700 real-time PCR instrument. Fluorescence values are typically given as a ratio between the fluorescence emitted by the molecular beacon (*e.g.,* labeled with FAM, HEX, or other fluorophores) and the ROX reference dye.

Numerous methods have been developed for detecting the products of RCA, *e.g.,* in solution, in emulsions, bound to beads or surfaces, *etc.* See, *e.g.,* US 7,862,999 to U.S. Pat. No. 6,316,229 to Lizardi, *et al*.; M. Ali, *et al.* "Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine". *Chemical Society Reviews*. **43** (10): 3324–3341, and U.S. Patent No. 11,186,863, each of which is incorporated herein by reference in its entirety, for all purposes.

In some embodiments, RCA is performed in the same buffer in which FEN cleavage and/or ligation occurs. In certain embodiments, a reaction mixture comprises target nucleic acids, padlock probes, FEN endonuclease, a ligase enzyme, and a DNA polymerase.

Identifying elements in ligatable molecules

In some embodiments, a circularizable molecule (*e.g.,* padlock probe, dumbbell probe, cleaved flaps) or linear vector or vector pair may comprise sequence elements that enable downstream analysis of the ligated molecule or the amplified product. For example, in some embodiments, padlock probes, dumbbell probes, linear vector oligonucleotides or vector pairs, or flap portions of flap oligonucleotides comprise encoded elements, unique molecular identifiers (UMI) sequences, one or more primer binding sites, *etc.,* which are generally useful for capturing, identifying, sequencing, and/or counting individual probes or flaps, or families of probes or flaps, that have been ligated as a result of the presence of a target nucleic acid, *e.g.*, in the presence of a cleaved flap. For example, in some embodiments, counting detected codes and estimating target quantity-based counts of detected codes, *e.g.,* finds use in estimating target quantity based on a correlation of counts of detected codes and target quantity in the sample analyzed. See, *e.g.,* U.S. Pat. No. 6,316,229 to Lizardi, which is incorporated herein by reference in its entirety for all purposes. In particularly preferred embodiments, the ligated molecules, *e.g.*, ligated padlock probes, dumbbell probes, linear ligation products of vector oligonucleotides or vector pairs, or circularized cleaved flaps are used as template for a DNA polymerase. In some embodiments, the ligated molecules are analyzed using a sequencing, preferably using next generation sequencing for parallel analysis, as discussed hereinabove.

Multiplexed detection of cleaved flaps

Provided herein is a method that utilizes endonuclease generated flap sequences as target identifiers, combined with next generation sequencing for ultra-sensitive and highly specific quantification of multiplexed PCR assays. An overview of an exemplary method described herein is shown in FIG. 9A, with an additional embodiment shown in FIG. 9B.

As shown in FIG. 9A, the initial step comprises an invasive cleavage assay (*e.g.,* PCR-flap assay). The invasive cleavage assay is performed using a flap oligonucleotide comprising a flap sequence and at least one target-specific region. The invasive cleavage assay may be performed on original samples comprising target nucleic acids, or may be performed, for example, on sample material that has been pre-amplified, preferably in a multiplexed amplification reaction, *e.g.*, as described in PCT/US23/66278, filed 04/27/2023, and in as is described in U.S. Pat. No. 10,648,025, and in WO 2020/112869, each of which is incorporated herein by reference in its entirety.

Such a flap oligonucleotide is shown in FIG. 9A as "sequence specific probe 1", and the flap sequence is shown in grey. The invasive cleavage assay is generally performed using a forward primer, which also serves as an invasive oligonucleotide or an invader oligonucleotide, a reverse primer, and a flap endonuclease (*e.g.,* FEN-1, preferably a strict FEN-1) to induce cleavage of the flap sequence.

The cleaved flap sequence is only generated upon binding of the flap oligonucleotide to a target and is therefore target-specific. The cleaved flap is referred to in FIG. 9A as a "cleaved marker-specific identifier arm", and can also be referred to herein as a "cleaved target-specific marker" or a "cleaved target specific-identifier." In addition to being a target-specific identifier, cleaved flap sequences may comprise subject-specific (*e.g.*, patient-specific) and/or sample-specific identifiers, *e.g.*, indexing sequences. In the absence of any particular target, the flap probe specific for that target is not cleaved and no cleaved flap is generated for that particular target.

As shown in FIG. 9A, following generation of the cleaved flap, the cleaved flap is ligated to a circularizable vector. In this exemplary method, the cleaved flap is ligated to the circularizable vector by hybridizing both the cleaved flap and the circularizable vector ends to a bridge oligonucleotide to form a ligatable nicked structure. The bridge oligonucleotide is referred to in the figure as the "arm specific bridging oligo."

Ligation of the cleaved flap to the circularizable vector forms a closed circular nucleic acid molecule containing the cleaved flap sequence (*e.g.,* containing the cleaved target-specific identifier). A library containing a plurality of such closed circular structures can be created by

amplification of a mixture of closed circular structures, thereby generating a sequencing library. The cleaved flap sequence, which is indicative of the target, can be detected by any suitable sequencing technique, including NGS (*e.g.,* Illumina sequencing). The methods described herein achieve a high-specificity initial multiplexed PCR reaction, followed by counting of generated target-specific identifiers (*e.g.,* cleaved flap sequences) by NGS, which greatly increases the number of simultaneously detected markers (*e.g.,* simultaneously detected target-specific cleaved flap sequences) compared to conventional qPCR.

As illustrated in Fig. 9B, the circularizable vector described above may further comprise a unique sequence, indicated in FIG. 9B as a "vector index sequence," thereby increasing the total indexing capacity of the system, enabling, for example, higher-level multiplexing in individual sequencing runs. Use of additional indexing sequences provides additional indexing capacity (*e.g.*, in addition to the indexing capacity based on amplification primer indexing), allowing higher level multiplexing of samples analyzed in, for example, a single sequencing run. In some embodiments, multiple vectors comprising different vector indexes may be used, *e.g.*, 2, 3, 4, 5, or more different indexing vectors.

EXPERIMENTAL

EXAMPLE 1

Multiplexed flap cleavage, ligation, and sequencing

Methods involving a preamplification reaction (*e.g.*, of multiple genetic targets), a secondary amplification reaction (*e.g.*, LQAS, TELQAS) to generate cleaved flaps, ligation of the cleaved flats into a cassette and indexing by PCR to generate sequencing libraries are referred to herein as Flap Ligation and Polymerization Sequencing (FLAP-seq). An exemplary method (*e.g.*, an exemplary FLAP-seq method) for producing a sequence library from highly multiplexed flap cleavage assays based on a PCR-flap assay reaction, *e.g.,* an LQAS reaction, as discussed above may comprise the following steps:

1) Preamplification
2) LQAS PCR-flap assay (preferably without a FRET cassette oligonucleotide)
3) Proteinase K treatment

4) Ligation (*e.g.,* HiFi Taq DNA Ligase)
5) Exonuclease treatment (*e.g.,* Exo I with Exo III)
6) Indexing PCR
7) AMPure XP treatment
8) Illumina Sequencing

1) Preamplification

Up to 50µL of extracted nucleic acid, *e.g.,* cfDNA from plasma, is combined with suitable primers/primer pairs and 1.875 units of GoTaq DNA polymerase in a reaction mixture of 25mM KCl, 7.5mM $MgCl_2$, 10mM MOPS, 0.008% Tween 20, 0.008% IGEPAL, 0.025%L BSA, 0.25mM dNTPs, pH7.5. The reaction mixture is cycled to 95°C for 5 minutes, followed by 95°C for 30 seconds and 64°C for 1 min, repeated for 12 cycles. An aliquot is diluted 1:10 with 10 mM Tris HCL, 0.1 mM EDTA.

2) LQAS PCR-flap assay

Aliquots of diluted preamplified mixture are amplified in an LQAS reaction comprising 10 µL of diluted preamp mixture and suitable primer pairs and flap oligonucleotides, with 0.05 units of GoTaq and 14.6 ng/uL of FEN-1 endonuclease (Afu FEN-1, Hologic, Inc.) in a reaction mixture of 10 mM MOPS, 0.3 mM Tris HCl, 7.5mM $MgCl_2$,0.8mM KCl, 0.008% Tween 20, 0.008% IGEPAL, 0.1 ug/uL BSA, 1.25% glycerol, 0.25mM dNTPs. The reaction mixture is cycled to 95°C for 3 minutes, followed by 92.5°C for 10 seconds and 62.5°C for 50 seconds, repeated for 27 cycles. An aliquot of the LQAS reaction mixture is diluted 1:10 in 10 mM Tris-HCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 10 mM KCl.

3) Proteinase K treatment

10 µL of the diluted LQAS mixture is treated with 2.5 units of Proteinase K at 37°C for 45 minutes followed by heat killing the proteinase at 95°C for 15 minutes.

4) Ligation

13 µL of the Proteinase K-treated sample is combined 6 µL of a mastermix of bridge oligonucleotides and the circularizable vector with 0.5 units of HiFi Taq DNA Ligase in 50mM

Tris-HCl, 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 1mM NAD, pH7.5. The mixture is incubated at 55°C for 1 minute, followed by 37°C for 6 minutes, repeated 10 cycles.

5) Exonuclease treatment

19 µL of the ligation mixture is combined with 3 µL of an exonuclease mixture comprising thermolabile Exonuclease I (1.71 units) and Exonuclease III (4.25 units). The mixture is incubated at 37°C for 90 minutes followed by 70°C for 30 min.

6) Indexing PCR

22µL of the reaction mixture from step 5 is combined with 53 µL of an indexing PCR mastermix comprising 1.875 units of GoTaq and indexing primers in a reaction mixture of 25mM KCl, 7.5mM $MgCl_2$, 10mM MOPS, 0.008% Tween 20, 0.008% IGEPAL, 0.025%L BSA, 0.25mM dNTPs, pH7.5. The reaction is incubated at 95°C for 5 minutes, followed by cycling at 95°C for 30 seconds and 64°C for 1 min, repeated 16 cycles.

7) PCR cleanup

Products of the indexing PCR are cleaned up to remove unincorporated dNTPs, primers, primer dimers, *etc., e.g.,* using AMPure XP bead-based reagent (Beckman Coulter Life Sciences), according to manufacturer's instructions.

8) Illumina Sequencing

Pooled final libraries are analyzed using the Illumina miSEQ procedure.

The skilled artisan will appreciate that, although the method is illustrated by reference to the Illumina NGS method, the method is readily adaptable to other methods of characterizing the cleaved flaps, including using other NGS methods.

EXAMPLE 2

Methods of Reducing effects of Residual DNA polymerase activity after PCR-flap assay

Additional experiments were conducted to determine the impact of modifying various parameters for the FLAP-seq assay.

The effects of dephosphorylation and phosphorylation (kinasing) flap assay products prior to ligation, and the use of different types of ligase in the preparation of sequencing libraries from cleaved flap sequences were tested.

Effects of phosphatase and kinase treatment of LQAS cleavage products

PCR-flap assays were conducted using LQAS conditions (without FRET cassettes) and three different methylated DNA markers (MDMs) target DNA. LQAS reaction conditions are further described in U.S. Pat. No. 10,648,025, and in WO 2020/112869. The experiment was conducted using 200 strands (2e2) of a calibrator or 20 strands of a calibrator (2e1) as positive controls, or 200 ng polyA as a no-template control (NTC). The LQAS flap assay products were treated with calf intestine alkaline phosphatase (CIP) to dephosphorylate residual dNTPs, followed by kinasing the products with polynucleotide kinase (PNK) to 5′ phosphorylate the cleaved flap molecules prior to the step of ligation to the circularizable molecule was compared to preparing ligated molecules without the CIP/PNK treatment.

The 300 pM arm-specific bridging oligonucleotide and phosphorylated circularizable molecule (the sequencing library vector) were used, and ligation was conducted as described above. After ligation, the reaction mixtures were processed as described above and, 20 cycles of indexing PCR were performed, and the products were sequenced. Read data from the sequencing is summarized in FIG. 10. These data show that, under all conditions, use of CIP and PNK significantly increased assay sensitivity, as indicated by increased number of reads for all three of the MDMs.

Additional options or improving assay sensitivity include, in some embodiments, using a 3′ terminal ribonucleotides on the bridge oligonucleotides, and mismatches between the bridge oligonucleotide and circularizable molecule may also be used as an alternative to, or in addition to, reduced ligation temperature, use of a bridge oligonucleotide with nucleotide modifications

that inhibit DNA polymerase activity, and/or use of CIP and PNK or Proteinase K treatments to improve assay sensitivity.

Exonuclease treatment

Non-ligated, non-circularized material (*e.g.,* circularizable molecules, residual primers, non-specific amplification products, *etc.*) can cause undesirable, non-specific signal in downstream assays. For example, non-ligated, non-circularized material may form primer-dimers, which are undesirably amplified to generate non-specific signal. To remove these products from a reaction mixture, *e.g.,* prior to the indexing PCR, treatment with one or more exonucleases can be performed after ligation.

Ligation reactions comprising LQAS reaction products and circularizable molecules were conducted as described above, but with the addition of an exonuclease treatment step added before indexing PCR. Ligation reactions comprised ratios and concentrations of bridge oligonucleotide:circularizable molecule (*e.g.,* splint:vector), over a 10-fold dilution series of splint:vector concentrations. 200 ng PolyA was used as no-target control nucleic acid. Exonuclease was added to the ligation reaction mixtures as described above and the products were amplified and quantified, with results shown in FIG. 11. As shown in FIG. 11, under all conditions the addition of the exonuclease treatment step reduced non-specific signal, including at the highest concentrations of bridge oligonucleotide:circularizable molecule (4000 pM) . These data show that exonuclease treatment reduces non-specific signal when no target is present.

Ligase fidelity

The use of high-fidelity ligation enzymes (having limited single stranded DNA ligation activity) is another means of minimizing or preventing unintended self-ligation of the vector molecules (circularizable molecules or vector pairs) in the absence of the cleaved flap oligonucleotides. Assays were conducted as described above, and the ligation mixtures were treated with T4 DNA ligase, HiFi Taq DNA Ligase, Taq DNA Ligase, or *E.coli* DNA ligase. Analysis of the ligated products is shown in Figs. 12A-12C.

FIG. 12A shows a bioanalyzer pseudogel of the final product after cleaved flap oligonucleotide ligation and circularized probe amplification enrichment. The top band (~285bp) is the intended target-specific product (ligation on the target strand) while the lower bands demonstrate unintended off-target products. The purity of on-target product generation correlates with the percent of reads that are mapped to the intended target flap sequence used as input.

FIG. 12B demonstrates differences in background reads generated in the no-ligase control ((-) Ligase) and no-template control (TE) reactions using different DNA ligases. Each ligase shows similar ligation efficiency between 1e8 and 1e11 strands of input.

FIG. 12C shows the difference in sequencing quality (generated using an Illumina MiSeq instrument) based on the DNA ligase used. The Phred quality score on the y-axis is graphed as a function of the position of the read (sequencing cycle number) on the x-axis.

These data show that use of high-fidelity ligase improves the purity of circularized probes containing the cleaved flap sequence, resulting in an increased percentage of reads mapping to flap sequences. The ligases with low single stranded DNA ligation activity (*e.g.,* HiFi Taq DNA ligase) also resulted in lower background reads and higher sequencing quality.

Bridge/splint oligonucleotide concentrations

A negative control splint (bridge) oligonucleotide (splint oligonucleotide with no corresponding cleaved flap added) was used to quantify non-specific reads (open bars) occurring in the absence of cleaved flap. Non-specific reads were compared to flap-specific reads using the methylated DNA marker (MDM) splints (closed bars). 200 strands of MDM template were used as input to measure flap-specific reads, using the methods described above. The results are shown in FIG. 13. These data show that use of appropriate splint oligonucleotide concentration in the nick ligation reaction has an effect on the number of non-specific reads. The splint oligonucleotide concentration thus is preferably chosen appropriately to maximize the amount of reads correctly mapping to the flap sequence, while minimizing non-specific reads.

EXAMPLE 3

Methods of ligating cleaved flaps using RNA bridging sequences

Many DNA polymerases, *e.g.*, Taq DNA polymerase, exhibit little or no ability to extend the 3′ end of an oligonucleotide primer on an RNA template strand. Thus, unwanted extension from the 3′ end of a ligatable nucleic acid hybridized to a bridge or splint oligonucleotide can be minimized by using an RNA bridge oligonucleotide. For example, in some embodiments a cleaved flap is ligated to a ligatable molecule, *e.g.*, a circularizable flap vector or a vector pair, using an RNA bridge oligonucleotide, or a chimeric bridge oligonucleotide in which at least a portion of the oligonucleotide strand comprises RNA, *e.g.*, as shown schematically in Fig. 14. During development of the FLAP-seq assay, it was found that the PBCV-1 DNA ligase was suitable for ligating DNA nicks using both RNA and DNA template strands. Advantageously, the PBCV-1 DNA ligase can ligate DNA nicks on unmodified RNA, 2'-O-methyl RNA, and chimerical bridging strands. See, *e.g.*, Lohman et al. US Pat. No. 10,597,650, which is incorporated herein by reference in its entirety. Use of an RNA or chimeric bridging oligonucleotide and a 5′ activatable flap vector reduces or removes the need to suppress the polymerase and to remove excess unligated flap vectors using multiple steps, resulting in a substantially shortened procedure comprising only the following steps:

1) Preamplification
2) LQAS PCR-flap assay
3) Ligation with RNA/chimeric bridge sequence (*e.g.*, SPLINTR ligase)
4) Indexing PCR
5) AMPure XP treatment
6) Illumina Sequencing

This method also resulted in reduced background in the assay.

EXAMPLE 4

Modulating reference signal by varying assay oligonucleotide concentrations

Flap Ligation and Polymerization Sequencing (FLAP-seq) was designed for multiplexed, targeted genomic interrogation, whether it be detection of methylation status, mutation detection or genotyping. Using FLAP-seq, multiple genetic targets can be co-amplified in a single preamplification reaction. This is followed by a secondary amplification reaction (*e.g.*, LQAS, TELQAS), in which probes with 5′ flaps are specifically and quantitatively cleaved to generate a population of cleaved flaps. Following the secondary amplification reaction, the cleaved flaps are ligated into a cassette and indexed using PCR to generate NGS libraries. In exemplary methods, the FLAP-Seq assay has a dynamic range up to ~2.5-3 orders of magnitude.

Reference nucleic acids, preferably nucleic acids that are comparably methylated in both normal and cancer samples (referred to as "universally methylated DNA markers") are used to normalize cancer-specific MDMs. Many cancer-specific MDMS produce signal falling within the linear phase of the FLAP-seq assays described herein. However, *B3GALT6*, the universal methylated MDM commonly used as a reference nucleic acid , can produce signal about 3 to 4 orders of magnitude higher than cancer-specific MDMs in plasma from individuals. Samples with high levels of *B3GALT6* DNA thus can fall outside the linear range of the FLAP-Seq assay described herein (FIG. 19). A separate LQAS assay for *B3GALT6* may be conducted, apart from a FLAP-Seq assay for the cancer-specific MDM quantification. However, this approach is disadvantageous due to increased cost, instrumentation, and time required to run two different assays.

As such, methods for attenuating *B3GALT6* amplification to shift *B3GALT6* signal into the linear range of the FLAP-Seq assay, to achieve multiplexed quantification of *B3GALT6* and cancer-specific MDMs were investigated herein. Amplification efficiency of *B3GALT6* was altered by titration of a single primer, both primers, the probe, or a combination of the three in either the preamplification, or the secondary amplification assay (*e.g.,* the TELQAS or the LQAS assay). As the objective was to reduce *B3GALT6* amplification efficiency, these oligonucleotides were used in reduced concentrations. Additionally, the design of a primer to include a single or

multiple mismatches between primer and template also reduced *B3GALT6* amplification efficiency.

An exemplary strategy for asymmetric downward titration of only the non-invading *B3GALT6* primer for amplification is shown in FIG. 18. However, it is expressly contemplated herein that the amplification efficiency may be reduced by any suitable method, including titration of the invading primer, titration of the non-invading primer, titration of the invading and the non-invading primer, titration of the probe (*e.g.*, the FLAP-oligonucleotide), or a combination thereof. Exemplary methods for titration of primers are described in Sint et al., Methods in Ecology and Evolution 2012, 3, 898-905 and in WO2005/038041A2, the contents of each of which are incorporated herein by reference in their entireties.

As shown in FIG. 20, the reduction in amplification efficiency of *B3GALT6* by reducing concentration of the non-invading *B3GALT6* primer by the indicated amounts resulted in reduced amplification in PCR-flap assays, delaying signal accumulation to shift the *B3GALT6* signal to a range similar to the cancer-specific MDMs. As shown in FIG. 21, reducing the concentration of *B3GALT6* invasive and non-invasive oligonucleotides reduced *BGALT6* amplification efficiency and shifted *B3GALT6* to within the linear range of the assay. These methods ensure that the cancer-specific and reference DNA markers fall within the linear range of the FLAP-Seq assay. Reducing the amplification efficiency of the methylated reference MDM is advantageous in that it allows both cancer-specific and reference MDMs to be quantified simultaneously, within the same FLAP-Seq reaction. This increases simplicity and reduces the cost compared to running separate reactions. Additionally, simultaneous quantification of the cancer-specific and reference MDMs within the same reaction more accurately normalizes for any run-to-run differences.

All literature and similar materials cited in this application, including but not limited to, patents, patent applications, articles, books, treatises, manufacturer publications and product literature, and internet web pages are expressly incorporated by reference in their entirety for any purpose. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which the various embodiments described herein belong. When definitions of terms in incorporated references

appear to differ from the definitions provided in the present teachings, the definition provided in the present teachings shall control.

Various modifications and variations of the described compositions, methods, and uses of the technology will be apparent to those skilled in the art without departing from the scope and spirit of the technology as described. Although the technology has been described in connection with specific exemplary embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in molecular biology, nucleic acid chemistry, biochemistry, medical science, or related fields are intended to be within the scope of the following claims.

Features, materials, characteristics, or groups described in conjunction with a particular aspect, embodiment, or example are to be understood to be applicable to any other aspect, embodiment or example described in this section or elsewhere in this specification unless incompatible therewith. All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive. The protection is not restricted to the details of any foregoing embodiments. The protection extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

Furthermore, certain features that are described in this disclosure in the context of separate implementations can also be implemented in combination in a single implementation. Conversely, various features that are described in the context of a single implementation can also be implemented in multiple implementations separately or in any suitable subcombination. Moreover, although features may be described above as acting in certain combinations, one or more features from a claimed combination can, in some cases, be excised from the combination, and the combination may be claimed as a subcombination or variation of a subcombination.

The following additional references are incorporated herein by reference in their entireties, for all purposes

1. Mu, J., *et al*., Plausibility of an extensive use of stool DNA test for screening advanced colorectal neoplasia. *Clin Chim Acta* **501**: p. 42-47 (2020).

2. Potter, N.T., *et al*., Validation of a real-time PCR-based qualitative assay for the detection of methylated SEPT9 DNA in human plasma. *Clin Che,* **60**(9): p. 1183-91 (2014).

3. Cohen, J.D., *et al*., Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* **359**(6378): p. 926-930 (2018).

4. Rodia, M.T., *et al*., LGALS4, CEACAM6, TSPAN8, and COL1A2: Blood Markers for Colorectal Cancer-Validation in a Cohort of Subjects With Positive Fecal Immunochemical Test Result. *Clin Colorectal Cancer,* **17**(2): p. e217-e228 (2018).

5. Chao, S., *et al*., Blood RNA biomarker panel detects both left- and right-sided colorectal neoplasms: a case-control study. *J Exp Clin Cancer Res* **32**: p. 44 (2013).

6. Ciarloni, L., *et al*., Discovery of a 29-gene panel in peripheral blood mononuclear cells for the detection of colorectal cancer and adenomas using high throughput real-time PCR. *PLoS One* **10**(4): p. e0123904 (2015).

7. *BCL Convert Conversion Software Support Resources*. 2022 January 27, 2022; Available on the world wide web at: support<dot>illumina<dot>com/sequencing/sequencing_software/bcl-convert

8. Patro, R., *et al*., Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods* **14**(4): p. 417-419 (2017).

9. Dobin, A., *et al*., STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, 2013. **29**(1): p. 15-21 (2013).

10. Broad Institute, *Picard*. 2022 [cited 2022 Jan 31,2022]; A set of command line tools (in Java) for manipulating high-throughput sequencing (HTS) data and formats such as SAM/BAM/CRAM and VCF.]. Available on the world wide web at broadinstitute<dot>github<dot>io/picard/.

11. Love, M.I., W. Huber, and S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **15**(12): p. 550 (2014).

12. T.M. Therneau, E.J.A., An introduction to recursive partitioning using the rpart routines, in Division of Biostatistics. *Mayo Clinic: Division of Biostatistics* (1997).

13. Naber, S.K., *et al*., Cost-effectiveness of a multitarget stool DNA test for colorectal cancer screening of Medicare beneficiaries. *PLoS One* **14**(9): p. e0220234 (2019).

14. Li, H., A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics* **27**(21): p. 2987-93 (2011).

15. Ensembl genome browser. *Ensembl87*. 2016; Available on the world wide web at: dec2016<dot>archive<dot>ensembl<dot>org/index.html.

16. Keller, L., *et al*., Clinical relevance of blood-based ctDNA analysis: mutation detection and beyond. *Br J Cancer* **124**(2): p. 345-358 (2021).

17. Phallen, J., *et al*., Direct detection of early-stage cancers using circulating tumor DNA. *Sci Transl Med* **9**(403) (2017).

18. Galamb, O., *et al*., Diagnostic mRNA expression patterns of inflamed, benign, and malignant colorectal biopsy specimen and their correlation with peripheral blood results. *Cancer Epidemiol Biomarkers Prev* **17**(10): p. 2835-45 (2008).

19. Kaiser M., *et al*. *J Biol Chem.* Vol 274, No 30, 21387-21394 (1999).

20. Dorjsuren D., *et al*. *Nucleic Acids Research* Vol 39, No 2 e11 (2011).

21. Lyamichev V., *et al*. *Biochemistry* 39, 9523-9532 (2000).

22. Tsutakawa S., *et al*. *Cell*. 145, 198-211 (2011).

23. Tumey N., *et al*. *Bioorg & Med Chem Let.* 15, 277-281 (2004).

24. Hall J.G., *et al*. Sensitive detection of DNA polymorphisms by the serial invasive signal amplification reaction. *Proc Natl Acad Sci USA* 97: 8272-8277 (2000).

25. Allawi H.T., *et al*. Invader Plus Method Detects Herpes Simplex Virus in Cerebrospinal Fluid and Simultaneously Differentiates Types 1 and 2. *J Clin Microbio* vol 44, no. 9: 3443-3447 (2006).

26. Ali, M. *et al.* Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine. *Chemical Society Reviews.* **43** (10): 3324–3341.

27. Nilsson, M., *et al*. Padlock probes: circularizing oligonucleotides for localized DNA detection. *Science*. 265 (5181): 2085–2088 (1994).

28. Banér, J., *et al*., Signal amplification of padlock probes by rolling circle replication. *Nucleic Acids Research* 26 (22): 5073–5078 (1998).

29. Huang, P.-J. J., and J. Liu, Molecular Beacon Lighting up on Graphene Oxide. *Anal. Chem.* 84:4192−4198 (2012).

30. Dahl, C.O.F., *et al.*, WO 2015/083002 Multiplex Detection of Nucleic Acids.

31. Head, S.R., *et al*., Library construction for next-generation sequencing: Overviews and challenges. *Biotechniques. 56(2): 61–passim (*2014).

32. Porreca, G.J., Genome sequencing on nanoballs. *Nature Biotechnology.* **28** (1): 43–4 (2010).

33. Yixiao Cui, *et al*., Cyclization of secondarily structured oligonucleotides to single-stranded rings by using *Taq* DNA ligase at high temperatures, *RSC. Adv.* 8:18972-79 (2018).

34. Tabuchi, I., *et al*., "An Efficient Ligation Method in the Making of an *in vitro* Virus for *in vitro* Protein Evolution" *Biol. Proced. Online* ;4(1): 49-54 (2002).

35. Szemes, M., *et al*., Diagnostic application of padlock probes—multiplex detection of plant pathogens using universal microarrays" *Nucleic Acids Research* 33(8): e70 (2005).

36. Dahl, C.O.F., *et al.*, US 10,526,643 B2 Multiplex Detection of Nucleic Acids.

37. Shapero, M., *et al.*, U.S. Pat. Pub. US 2015/0284786 Al "Compositions and methods for molecular inversion probe assays.

38. Hardenbol, *et al.*, Multiplexed genotyping with sequence-tagged molecular inversion probes. *Nature Biotechnology* v21(6):673 (2003).

39. Mohsen, M. *et al.*, The Discovery of Rolling Circle Amplification and Rolling Circle Transcription, *Acc Chem Res.* 2016 November 15; 49(11): 2540–2550 (2016).

40. Sorge, J.A., Methods for detection of a nucleic acid by sequential amplification,

US Pat. No. 7,309,573

41. V. Lyamichev, *et al*., Polymorphism identification and quantitative detection of genomic DNA by invasive cleavage of oligonucleotide probes. *Nature Biotechnology* v17, p292 (1999).

42. Prakash G, Kool ET. Molecular Recognition by Circular Oligonucletides: Strong Binding of Single- and Double-stranded DNA and RNA. *J Chem Soc, Chem Commun.* 1161–1163 (1991).

43. Kool ET. Molecular Recognition by Circular Oligonucleotides: Increasing the Selectivity of DNA Binding. *J Am Chem Soc.* 113:6265–6266 (1991).

44. Diegelman AM, Kool ET. Chemical and Enzymatic Methods for Preparing Circular Single-Stranded DNAs. *Curr Protoc Nucleic Acid Chem.* 5.2.1–5.2.27 (2000).

45. Kool E.T. Recognition of DNA, RNA, and proteins by circular oligonucleotides. *Acc Chem Res.* 31:502–510. (1998)

46. Kool E.T. Circular DNA vectors for synthesis of RNA and DNA. US Pat. No. 7,906,490.

47. Kool E.T. Highly sensitive multimeric nucleic acid probes. US Pat. No. 6077668.

48. Kool E.T. Rolling circle synthesis of oligonucleotides and amplification of select randomized circular oligonucleotides. US Pat. No. 5,714,320.

49. US Pat. No. 7,045,289 to Allawi, *et al*.

50. Krzywkowski, T. and M. Nilsson, Fidelity of RNA templated end-joining by chlorella virus DNA ligase and a novel iLock assay with improved direct RNA detection accuracy *Nucleic Acids Research,* Vol. 45, No. 18:e16151 (2017).

51. Shin, M et al., Quantification of Antisense Oligonucleotides by Splint Ligation and Quantitative Polymerase Chain Reaction; *Nucleic Acids Therapeutics* v32 (1):66-72 (2022).

CLAIMS

What is claimed is:

1. A method comprising:

a) providing a reaction mixture comprising or suspected of comprising at least one target nucleic acid, at least one flap oligonucleotide, and a flap endonuclease, wherein each flap oligonucleotide comprises:

i) a target-specific portion that anneals to a target nucleic acid, if the target nucleic acid is present in the reaction mixture, and

ii) a 5′ flap portion, comprising a sequence of nucleotides indicative of the target nucleic acid to which the target-specific portion anneals;

wherein, for target nucleic acids present in the reaction mixture, invasive cleavage structures comprising a target nucleic acid annealed to a flap oligonucleotide are formed;

wherein, in the reaction mixture, invasive cleavage structures are cleaved by the flap endonuclease to produce at least one cleaved flap;

b) providing at least one ligated molecule, wherein the ligated molecule comprises sequence of the cleaved flap, and/or a sequence complementary to the cleaved flap, and

c) characterizing the at least one ligated molecule to characterize the at least one target nucleic acid present in the reaction mixture.

2. A method comprising:

a) providing a reaction mixture comprising or suspected of comprising a population of different target nucleic acids, a plurality of flap oligonucleotides, and a flap endonuclease, wherein each flap oligonucleotide comprises:

i) a target-specific portion that anneals to a target nucleic acid, if the target nucleic acid is present in the reaction mixture, and

ii) a 5′ flap portion, comprising a sequence of nucleotides indicative of the target nucleic acid to which the target-specific portion anneals;

wherein, for target nucleic acids present in the reaction mixture, invasive cleavage structures comprising a target nucleic acid annealed to a flap oligonucleotide are formed;

wherein, in the reaction mixture, the invasive cleavage structures are cleaved by the flap endonuclease to produce population of cleaved flaps;

b) providing a population of ligated molecules, wherein the ligated molecules comprise sequences of the population of cleaved flaps, and/or sequences complementary to the population of cleaved flaps, and

c) characterizing the population of ligated molecules to characterize the population of different target nucleic acids present in the reaction mixture.

3. The method of any one of claims 1-2, wherein providing the ligated molecule comprises treating the cleaved flap with a preparation comprising a ligatable vector molecule under conditions wherein a cleaved flap is ligated to the ligatable vector molecule to form a ligated molecule, each ligated molecule comprising a vector molecule sequence and a cleaved flap sequence; and/or, wherein the reaction mixture is an amplification reaction mixture, and wherein the at least one target nucleic acid comprises at least one amplified nucleic acid produced in the amplification reaction mixture; preferably, wherein the amplification reaction mixture is a PCR-flap assay reaction mixture; and/or, wherein characterizing the at least one ligated molecule comprises counting different cleaved flap sequences, or complements thereof, if present in the reaction mixture; preferably, wherein characterizing the at least one ligated molecule comprises sequencing cleaved flap sequences, or the complements thereof, if present in the reaction mixture; and/or, wherein the flap endonuclease is a FEN-1 endonuclease; preferably, wherein the FEN-1 endonuclease is a strict FEN-1 endonuclease, and wherein the reaction mixture comprises a Mg^{++} flap assay buffer; preferably, wherein the strict FEN-1 endonuclease is selected from the group consisting of *Archaeoglobus fulgidus* (Afu) FEN-1, *Pyrococcus furiosus* (Pfu) FEN-1, and *Archaeoglobus veneficus* (Ave) FEN-1, and variants thereof, and CLEAVASE 2.0 nuclease.

4. The method of any one of claims 1-3, wherein each ligated molecule comprises one or more of:

i) a first bridge oligonucleotide-complementary arm at a 3′ end of a first vector sequence and a second bridge oligonucleotide-complementary arm at a 5′ end of a second vector sequence, wherein the first bridge oligonucleotide-complementary arm has a different nucleotide sequence than the second bridge oligonucleotide-complementary arm;

ii) a pair of amplification primer binding sites configured to amplify a portion of a ligated molecule that comprises a cleaved flap sequence;

iii) a primer binding site configured to amplify a circular ligated molecule by rolling circle amplification;

iv) at least one unique molecular tag;

v) a polynucleotide linker; and/or

vi) a polymerization-terminating molecule, preferably an internal spacer;

and/or, wherein the method further comprises a step of amplifying a region of the at least one ligated molecule comprising a cleaved flap sequence to form at least one flap amplicon; preferably, further comprising sequencing the at least one flap amplicon to produce sequencing reads.

5. The method of any one of claims 3-4, wherein the ligatable vector molecule is selected from a dumbbell probe, a padlock probe, a vector pair, and/or a linear vector molecule; and/or, wherein the ligatable vector molecule comprises a vector pair comprising a first bridge oligonucleotide-complementary arm at a 3′ end of a first vector sequence and a second bridge oligonucleotide-complementary arm at a 5′ end of a second vector sequence, and wherein step b) comprises contacting the at least one cleaved flap with the ligatable vector molecule and a bridge oligonucleotide, wherein the bridge oligonucleotide comprises an inner segment that is complementary to a cleaved flap sequence, a 3′ end that is complementary to the 3′ end of the first vector sequence, and a 5′ end that is complementary to the 5′ end of the second vector sequence;

preferably, wherein the ligatable vector molecule comprising the 5′ end of the second vector sequence comprises a 5′ blocking group; and/or, wherein the ligatable vector molecule is a dumbbell probe comprising a bridge sequence that is complementary to a cleaved flap sequence.

6. The method of any one of claims 1-5, wherein the target-specific portions of the flap oligonucleotide in the reaction mixture is at least 6 nucleotides in length, preferably at least 7, preferably at least 8, preferably at least 9, preferably at least 10, preferably at least 11, preferably at least 12 nucleotides in length; preferably, wherein the target-specific portion of the flap oligonucleotide in the reaction mixture is 13 to 100 nucleotides in length; and/or wherein the target nucleic acid comprises one or more of cDNA, amplified DNA, genomic DNA, and circulating cell-free DNA; and/or, wherein the target nucleic acid comprises DNA treated with a methylation-specific reagent; preferably, wherein the reaction mixture further comprises oligonucleotides specific for a reference nucleic acid, the reference nucleic acid preferably comprising methylated *B3GALT6* DNA; preferably, wherein the oligonucleotides comprise a flap oligonucleotide, an invasive oligonucleotide, and optionally a non-invasive oligonucleotide; and/or, wherein a concentration of one or more of the oligonucleotides specific for the reference nucleic acid is altered in the reaction mixture relative to a concentration of corresponding oligonucleotides specific for the target nucleic acid in the reaction mixture, and/or wherein one or more of the oligonucleotides specific for the reference nucleic acid binds to the reference nucleic acid with one or more mismatched base pairings; preferably, wherein the concentration of the one or more of the oligonucleotides specific for the reference nucleic acid is reduced in the reaction mixture relative to the concentration of the corresponding oligonucleotides specific for the target nucleic acid in the reaction mixture.

7. The method of any one of claims 1-6, wherein the ligated molecule is a circular molecule.

8. The method of claim 7, wherein characterizing the circular molecule comprises detecting hybridization of at least one cleaved flap to the circular molecule, or wherein providing the circular molecule comprises treating the at least one cleaved flap under conditions wherein 5′ and 3′ ends

of individual cleaved flaps are ligated together to form a circular molecule; or, wherein each circularizable molecule comprises a pair of bridge oligonucleotide-complementary arms at 5′ and 3′ ends of the circularizable molecule, and wherein step b) comprises contacting the at least one cleaved flap with the circularizable molecule and a bridge oligonucleotide, wherein the bridge oligonucleotide comprises an inner segment that is complementary to a cleaved flap sequence, a 5′ end complementary to the 5′ end of the circularizable molecule, and a 3′ end that is complementary to a 3′ end of the circularizable molecule; and/or, the method further comprising a step of replicating the circular molecule comprising cleaved flap sequence using rolling circle replication, to form a flap replicon.

9. The method of any one of claims 1-6, wherein the ligated molecules are linear molecules.

10. The method of any one of claims 2-9, wherein the plurality of flap oligonucleotides in a reaction mixture comprises at least 10 unique flap oligonucleotides; preferably, wherein the plurality of flap oligonucleotides in a reaction mixture comprises at least 20 unique flap oligonucleotides; or, wherein the plurality of flap oligonucleotides in a reaction mixture comprises at least 30 unique flap oligonucleotides; or , wherein the plurality of flap oligonucleotides in a reaction mixture comprises 40 to 1000 unique flap oligonucleotides.

11. A composition comprising:

i) one or both of:

a) at least one cleaved flap; and

b) at least one ligated molecule comprising a cleaved flap sequence or a complement thereof, wherein the cleaved flap sequence or complement thereof comprises a sequence of nucleotides indicative of a target nucleic acid;

and

ii) a ligase enzyme.

12. A composition comprising:

i) one or both of:

a) a population of cleaved flaps; and

b) a population of ligated molecules, each ligated molecule comprising a cleaved flap sequence or a complement thereof, wherein each cleaved flap sequence or complement thereof comprises a sequence of nucleotides indicative of a target nucleic acid;

and

ii) a ligase enzyme.

13. The composition of any one of claims 11-12, further comprising at least one bridge oligonucleotide, wherein each bridge oligonucleotide comprises a 5′ end sequence that is complementary to a 5′ end of a cleaved flap sequence, and a 3′ end sequence that is complementary a 3′ end of the cleaved flap sequence, wherein hybridization of the bridge oligonucleotide to a cleaved flap, if present in the composition, forms a ligatable nick; preferably, further comprising at least one ligatable vector molecule, wherein each ligated molecule comprises at least one ligatable vector molecule sequence; and/or, further comprising at least one bridge oligonucleotide, wherein each bridge oligonucleotide comprises an inner segment that is complementary to a cleaved flap sequence, a 3′ end that is complementary to a 3′ end of a first vector sequence, and a 5′ end that is complementary to the 5′ end of a second vector sequence; preferably, further comprising one or more of the following:

i) a first ligatable vector molecule and second ligatable vector molecule, the second ligatable vector molecule comprising a 5′ blocking group, wherein in the composition, the bridge oligonucleotide is hybridized to the cleaved flap and to the first and second ligatable vector molecules in a complex comprising:

I) a first ligatable nick comprising the 3′ end of the first ligatable molecule and a 5′ end of the cleaved flap, and

II) an invasive cleavage substrate comprising a 3′ end of the cleaved flap and the 5′ end of the second ligatable vector molecule comprising the 5′ blocking group,

wherein the 5′ blocking group preferably comprises a moiety selected from a hexane, a non-standard nucleotide, an abasic nucleotide, a fluorophore, a flap nucleic acid sequence, a hairpin flap nucleic acid sequence, and a hydroxyl group;

ii) a bridge oligonucleotide comprising one or more of an RNA portion and a DNA portion;

iii) a bridge oligonucleotide comprising an RNA portion comprising one or more nucleotide modifications, preferably selected from 2′-O-methyl and 2′-O-methoxyethyl nucleotide modifications;

iv) a flap endonuclease, preferably a FEN-1 endonuclease, preferably a strict FEN-1 endonuclease, preferably a strict FEN-1 endonuclease selected from the group consisting of *Archaeoglobus fulgidus* (Afu) FEN-1, *Pyrococcus furiosus* (Pfu) FEN-1, and *Archaeoglobus veneficus* (Ave) FEN-1, and variants thereof, and CLEAVASE 2.0 nuclease;

v) a Mg^{++} flap assay buffer;

vi) amplification reagents;

vii) flap assay reagents; and/or

viii) a ligase comprising *Chlorella* virus PBCV-1 DNA ligase;

ix) a ligated molecule comprising one or more of:

a) a pair of amplification primer binding sites configured to amplify a portion of a ligated molecule that comprises a cleaved flap sequence;

b) a primer binding site configured to amplify a circular ligated molecule by rolling circle amplification;

c) at least one unique molecular tag;

d) a polynucleotide linker; and/or

e) a polymerization-terminating molecule, preferably an internal spacer;

and/or,

x) at least one amplified product, each amplified product comprising a target nucleic acid sequence that is associated with a cleaved flap sequence in a ligated molecule.

14. The composition of any one of claims 11-13, wherein the ligated molecule is a circular molecule.

15. A method, comprising:

a) providing in a mixture a bridge oligonucleotide, a flap sequence oligonucleotide, preferably a cleaved flap oligonucleotide, a first ligatable vector molecule and second ligatable vector molecule comprising a 5′ blocking group, wherein the bridge oligonucleotide comprises:

i) an inner segment that is complementary to the flap sequence oligonucleotide,

ii) a 3′ end that is complementary to a 3′ end of the first ligatable vector molecule, and

iii) a 5′ end that is complementary to a 5′ end of the second ligatable vector molecule,

wherein, in the mixture, the bridge oligonucleotide is hybridized to the flap sequence oligonucleotide and to the first and second ligatable molecules to form a complex comprising:

I) a first ligatable nick comprising the 3′ end of the first ligatable molecule and a 5′ end of the flap sequence oligonucleotide, and

II) an invasive cleavage substrate comprising a 3′ end of the flap sequence oligonucleotide and the 5′ end of the second ligatable vector molecule comprising the 5′ blocking group;

b) treating the complex with a flap endonuclease in conditions wherein cleavage of the invasive cleavage substrate cleaves off the 5′ blocking group of the second ligatable vector molecule and produces a second ligatable nick; and

c) treating the complex with a ligase under conditions wherein the first ligatable nick and the second ligatable nick are ligated to form a ligated molecule.

16. The method of claim 15, wherein the 5′ blocking group comprises a moiety selected from a hexane, a non-standard nucleotide, an abasic nucleotide, a fluorophore, a flap nucleic acid sequence, a hairpin flap nucleic acid sequence, and a hydroxyl group; and/or, wherein the bridge oligonucleotide comprises one or more of an RNA portion and a DNA portion; preferably, wherein the RNA portion comprises one or more nucleotide modifications; preferably, , wherein the one or more nucleotide modifications comprise 2′-O-methyl or 2′-O-methoxyethyl modifications; and/or, wherein the 5′ end and an adjacent part of the inner segment of the bridge oligonucleotide comprise RNA, 2′-O-methyl RNA, or 2′-O-methoxyethyl RNA; preferably, wherein the 3′ end and an adjacent part of the inner segment of the bridge oligonucleotide comprise DNA; and/or, further comprising detecting the ligated molecule in a nucleic acid detection assay.