

CLAIMS

What is claimed is:

1. A method of improving specificity during amplification of a target DNA sequence, comprising:

(a) providing a reaction mixture, comprising:

(i) an oligonucleotide primer having a cleavage domain, which is cleavable by ~~an~~ a thermophilic, type II RNase H enzyme, positioned 5' of a blocking group, said blocking group linked at or near the 3'-end of the oligonucleotide primer wherein said blocking group prevents primer extension and/or inhibits the oligonucleotide primer from serving as a template for DNA synthesis,

(ii) a sample nucleic acid that may or may not the target sequence,

(iii) a DNA polymerase, and

(iv) ~~an~~ a thermophilic, type II RNase H enzyme wherein said thermophilic, type II RNase H enzyme is thermostable and has at least a 10-fold decrease in activity at 30°C as compared to 70°C,

(b) hybridizing the oligonucleotide primer to the target DNA sequence to form a double-stranded substrate;

(c) cleaving the hybridized oligonucleotide primer with said thermophilic, type II RNase H enzyme at a cleavage site within or adjacent to the cleavage domain to remove the blocking group from the oligonucleotide primer; and

(d) extending the oligonucleotide primer with the DNA polymerase, wherein the reaction mixture includes a concentration of a divalent cation such that a ΔC_p of at least about 5.0 or greater or about 50% or greater is obtained for the amplification of the target DNA as adjudged by a 3'-mismatch discrimination assay.

2. The method of claim 1, wherein the divalent cation is selected from Mg^{++} , Mn^{++} and Co^{++} .

3. The method of claims 1 or 2, wherein the divalent cation comprises Mg^{++} .

4. The method of claim 3, wherein a final concentration of Mg^{++} comprises about 2.0 mM free Mg^{++} or less, provided that an amplification of the target DNA sequence occurs.
5. The method of claim 3, wherein the reaction mixture further comprises a divalent cation different from Mg^{++} .
6. The method of claim 5, wherein the divalent cation different from Mg^{++} is selected from Mn^{++} , Co^{++} or a combination of Mn^{++} and Co^{++} .
7. The method of any of the preceding claims, wherein the thermophilic, type II RNase H enzyme is an RNase H2 enzyme.
8. The method of claim 7, wherein the RNase H2 enzyme is *Pyrococcus abyssi* RNase H2, *Pyrococcus kodakaraensis* RNase H2, *Pyrococcus furiosus* RNase H2, *Methanococcus jannaschii* RNase H2, or *Sulfolobus solfataricus* RNase H2.
9. The method of claim 7, wherein the RNase H2 enzyme is encoded by any one of SEQ ID NOS: 1-5.
10. The method of claim 7, wherein the RNase H2 enzyme is *Pyrococcus abyssi* RNase H2.
11. The method of claim 10, wherein the *Pyrococcus abyssi* RNase H2 is encoded by SEQ ID NO: 4.
12. The method of any of the preceding claims, wherein the thermophilic, type II RNase H enzyme is reversibly inactivated either by chemical modification or by a blocking antibody.
13. The method of any of the preceding claims, wherein the blocking group is attached to the 3'-terminal nucleotide of the oligonucleotide primer.

14. The method of any of the preceding claims, wherein the blocking group is attached 5' of the 3'-terminal residue and inhibits the oligonucleotide primer from serving as a template for DNA synthesis.
15. The method of claim 14, wherein the blocking group includes one or more abasic residues.
16. The method of claim 15, wherein the one or more abasic residues is a C3 spacer.
17. The method of any of the preceding claims, wherein the blocking group comprises one member selected from the group consisting of RDDDDx, RDDDDMx, RDxxD, RDxxDM, RDDDDxxD, RDDDDxxDM and DxxD, wherein R is an RNA residue, D is a DNA residue, M is a mismatched residue and x is a C3 spacer.
18. The method of any of the preceding claims, wherein the blocking group includes a label permitting detection of the extension amplification reaction.
19. The method of any of the preceding claims, wherein a label permitting detection of the amplification reaction is attached to the oligonucleotide primer 3' from the cleavage site.
20. The method of claims 18 or 19, wherein the label is a fluorophore.
21. The method of claims 18 or 19, wherein the label is a mass tag for detection of the amplification reaction by mass spectrometry.
22. The method of any of the preceding claims, wherein the cleavage domain comprises one or more of the following moieties: a DNA residue, an abasic residue, a modified nucleoside, or a modified phosphate internucleotide linkage.
23. The method of any of the preceding claims, wherein the cleavage domain comprises a single RNA residue.

24. The method of any of the preceding claims, wherein the cleavage domain comprises two adjacent RNA residues.
25. The method of any of the preceding claims, wherein the cleavage domain comprises a continuous sequence of three or more RNA residues.
26. The method of any of the preceding claims, wherein the cleavage domain lacks an RNA residue.
27. The method of any of the preceding claims, wherein the cleavage domain comprises one or more 2'-modified nucleosides.
28. The method of claim 27, wherein the one or more 2'-modified nucleosides is selected from the group consisting of 2'-O-alkyl RNA nucleoside, 2'-fluoronucleoside, locked nucleic acid, 2'- ethylene nucleic acid residue, 2'-alkyl nucleoside, 2' - aminonucleoside and 2'-thionucleoside.
29. The method of claim 27, wherein the one or more 2'-modified nucleosides is 2'-O-methyl RNA nucleoside.
30. The method of claim 27, wherein the one or more 2'-modified nucleosides is 2'-fluoronucleoside.
31. The method of any of the preceding claims, wherein a sequence flanking the cleavage site contains one or more internucleoside linkages resistant to nuclease cleavage.
32. The method of claim 31, wherein the nuclease resistant linkage is a phosphorothioate.
33. The method of any of the preceding claims, wherein the first and second reaction mixtures include a non-ionic detergent.

34. The method of claim 33, wherein the non-ionic detergent comprises polyethylene glycol hexadecyl ether.

35. The method of claim 34, wherein polyethylene glycol hexadecyl ether is present at a final concentration of at least about 0.001% in the first and second reaction mixtures.

36. A method of amplifying a target DNA sequence, comprising:

(a) providing a reaction mixture comprising:

(i) an oligonucleotide primer having a cleavage domain, which is cleavable by ~~an~~ a thermophilic, type II RNase H enzyme, positioned 5' of a blocking group, said blocking group linked at or near the 3'-end of the oligonucleotide primer wherein said blocking group prevents primer extension and/or inhibits the oligonucleotide primer from serving as a template for DNA synthesis,

(ii) a sample nucleic acid that may or may not the target sequence,

(iii) a DNA polymerase, and

(iv) ~~an~~ a thermophilic, type II RNase H enzyme wherein said thermophilic, type II RNase H enzyme is thermostable and has at least a 10-fold decrease in activity at 30°C as compared to 70°C,

(b) hybridizing the oligonucleotide primer to the target DNA sequence to form a double-stranded substrate;

(c) cleaving the hybridized oligonucleotide primer with said thermophilic, type II RNase H enzyme at a cleavage site within or adjacent to the cleavage domain to remove the blocking group from the oligonucleotide primer; and

(d) extending the oligonucleotide primer with the DNA polymerase, wherein the reaction mixture includes a divalent cation and a non-ionic detergent comprising polyethylene glycol hexadecyl ether.

37. The method of claim 36, wherein polyethylene glycol hexadecyl ether is present at a final concentration of at least about 0.001% in the reaction mixture.

38. The method of claims 36 or 37, wherein the divalent cation is selected from Mg^{++} , Mn^{++} and Co^{++} .
39. The method of claims 36, 37 or 38, wherein the divalent cation comprises Mg^{++} .
40. The method of any of claims 36-39, wherein the reaction mixture comprises a final concentration of Mg^{++} of about 2.0 mM free Mg^{++} or less, provided that an amplification of the target DNA sequence occurs.
41. The method of any of claims 36-40, wherein the reaction mixture further comprises a divalent cation different from Mg^{++} .
42. The method of claim 41, wherein the divalent cation different from Mg^{++} is selected from Mn^{++} , Co^{++} or a combination of Mn^{++} and Co^{++} .
43. The method of any of claims 36-42, wherein the thermophilic, type II RNase H enzyme is an RNase H2 enzyme.
44. The method of claim 43, wherein the RNase H2 enzyme is *Pyrococcus abyssi* RNase H2, *Pyrococcus kodakaraensis* RNase H2, *Pyrococcus furiosus* RNase H2, *Methanococcus jannaschii* RNase H2, or *Sulfolobus solfataricus* RNase H2.
45. The method of claim 43, wherein the RNase H2 enzyme is encoded by any one of SEQ ID NOS: 1-5.
46. The method of claim 43, wherein the RNase H2 enzyme is *Pyrococcus abyssi* RNase H2.
47. The method of claim 46, wherein the *Pyrococcus abyssi* RNase H2 is encoded by SEQ ID NO: 4.

48. The method of any of claims 36-47, wherein the thermophilic, type II RNase H enzyme is reversibly inactivated either by chemical modification or by a blocking antibody.

49. The method of any of claims 36-48, wherein the blocking group is attached to the 3'-terminal nucleotide of the oligonucleotide primer.

50. The method of any of claims 36-49, wherein the blocking group is attached 5' of the 3'-terminal residue and inhibits the oligonucleotide primer from serving as a template for DNA synthesis.

51. The method of any of claim 36-50, wherein the blocking group includes one or more abasic residues.

52. The method of claim 51, wherein the one or more abasic residues is a C3 spacer.

53. The method of any of claims 36-52, wherein the blocking group comprises one member selected from the group consisting of RDDDDx, RDDDDMx, RDxxD, RDxxDM, RDDDDxxD, RDDDDxxDM and DxxD, wherein R is an RNA residue, D is a DNA residue, M is a mismatched residue and x is a C3 spacer.

54. The method of any of claims 36-53, wherein the blocking group includes a label permitting detection of the extension amplification reaction.

55. The method of any of claims 36-54, further comprising a label permitting detection of the amplification reaction, wherein the label is attached to the oligonucleotide primer 3' from the cleavage site.

56. The method of claim 54 or 55, wherein the label is a fluorophore.

57. The method of claim 54 or 55, wherein the label is a mass tag for detection of the amplification reaction by mass spectrometry.

58. The method of any of claims 36-57, wherein the cleavage domain comprises one or more of the following moieties: a DNA residue, an abasic residue, a modified nucleoside, or a modified phosphate internucleotide linkage.
59. The method of any of claims 36-58, wherein the cleavage domain comprises a single RNA residue.
60. The method of any of claims 36-59, wherein the cleavage domain comprises two adjacent RNA residues.
61. The method of any of claims 36-60, wherein the cleavage domain comprises a continuous sequence of three or more RNA residues.
62. The method of any of claims 36-61, wherein the cleavage domain lacks an RNA residue.
63. The method of any of claims 36-62, wherein the cleavage domain comprises one or more 2'-modified nucleosides.
64. The method of claim 63, wherein the one or more 2'-modified nucleosides are selected from the group consisting of 2'-O-alkyl RNA nucleoside, 2'-fluoronucleoside, locked nucleic acid, 2'-ethylene nucleic acid residue, 2'-alkyl nucleoside, 2'-aminonucleoside and 2'-thionucleoside.
65. The method of claim 63, wherein the one or more 2'-modified nucleosides is 2'-O-methyl RNA nucleoside.
66. The method of claim 63, wherein the one or more 2'-modified nucleosides is 2'-fluoronucleoside.
67. The method of any of claims 36-66, wherein a sequence flanking the cleavage site contains one or more internucleoside linkages resistant to nuclease cleavage.

68. The method of claim 67, wherein the nuclease resistant linkage is a phosphorothioate.

~~69. A kit for performing amplification of a target DNA sequence, comprising a reaction buffer that includes a metal salt comprising a divalent cation and associated counterion, wherein the divalent cation comprises Mg^{++} , and the reaction buffer provides a final concentration of the metal salt no greater than about 2.0 mM free Mg^{++} in the reaction mixture for performing amplification of the target DNA.~~

~~70. The kit of claim 69, further comprising a non-ionic detergent.~~

~~71~~69. ~~The kit of claims 69 or 70,~~ A kit for performing amplification of a target DNA sequence, comprising a reaction buffer that includes a metal salt comprising a divalent cation and associated counterion, wherein the divalent cation comprises Mg^{++} , and the reaction buffer provides a final concentration of the metal salt no greater than about 2.0 mM free Mg^{++} in the reaction mixture for performing amplification of the target DNA and a non-ionic detergent, wherein the non-ionic detergent comprises polyethylene glycol hexadecyl ether.

~~72~~70. The kit of claim ~~71~~ 69, wherein polyethylene glycol hexadecyl ether is present at a final concentration of at least about 0.001% in the reaction mixture for performing amplification of the target DNA.

~~73~~71. The kit of ~~any of claims 69-72~~ or 70, wherein the reaction mixture further comprises at least one metal salt comprising a divalent cation different from Mg^{++} .

~~74~~72. The kit of claim ~~73~~ 71, wherein the divalent cation different from Mg^{++} is selected from Mn^{++} , Co^{++} or a combination of Mn^{++} and Co^{++} .

~~75~~73. The kit of any of claims 69-~~74~~72, further comprising
- one or more oligonucleotide primers, wherein at least one oligonucleotide primer has a cleavage domain, which is cleavable by ~~an~~ a thermophilic, type II RNase

H enzyme, positioned 5' of a blocking group, said blocking group linked at or near the 3'-end of the oligonucleotide primer wherein said blocking group prevents primer extension and/or inhibits the oligonucleotide primer from serving as a template for DNA synthesis,

- a DNA polymerase, and

- ~~an~~ a thermophilic, type II RNase H enzyme wherein said thermophilic, type II RNase H enzyme is thermostable and has at least a 10-fold decrease in activity at 30°C as compared to 70°C,

~~76~~74. The kit of claim~~-75~~ 73, wherein the RNase H enzyme is an RNase H2 enzyme.

~~77~~75. The kit of claim~~-76~~ 74, wherein the RNase H2 enzyme is *Pyrococcus abyssi* RNase H2, *Pyrococcus kodakaraensis* RNase H2, *Pyrococcus furiosus* RNase H2, *Methanococcus jannaschii* RNase H2, or *Sulfolobus solfataricus* RNase H2.

~~78~~76. The kit of claim~~-76~~ 74, wherein the RNase H2 enzyme is encoded by any one of SEQ ID NOS: 1-5.

~~79~~77. The kit of claim~~-76~~ 74, wherein the RNase H2 enzyme is *Pyrococcus abyssi* RNase H2.

~~80~~78. The kit of claim~~-79~~ 77, wherein the *Pyrococcus abyssi* RNase H2 is encoded by SEQ ID NO: 4.

~~81~~79. The kit of any of claims~~-75-80~~ 73-78, wherein the blocking group comprises one member selected from the group consisting of RDDDDx, RDDDDMx, RDxxD, RDxxDM, RDDDDxxD, RDDDDxxDM and DxxD, wherein R is an RNA residue, D is a DNA residue, M is a mismatched residue and x is a C3 spacer.