

Claims

1. A method for detecting a target nucleotide sequence comprising:
 - a) tagging the target nucleotide sequence with a nucleotide tag sequence, thereby producing a tagged target nucleic acid sequence;
 - b) providing a probe oligonucleotide comprising a nucleotide tag recognition sequence complementary to the nucleotide tag sequence and a regulatory sequence 5' to the nucleotide tag recognition sequence, wherein said probe oligonucleotide comprises a first label and has a melting temperature T_{m1} ;
 - c) amplifying the tagged target nucleic acid sequence in a PCR amplification reaction using the probe oligonucleotide as a primer, wherein said PCR amplification reaction is characterized by an annealing temperature T_a ;
wherein the PCR amplification reaction is carried out in the presence of a regulatory oligonucleotide comprising a sequence segment that is complementary to the regulatory sequence, wherein said regulatory oligonucleotide comprises a second label and has a melting temperature T_{m2} ; and
 - d) detecting the product of the PCR amplification reaction;
wherein the first label and the second label constitute a fluorescent reporter/quencher pair; and
wherein T_{m1} and T_{m2} are both higher than T_a .
2. The method of claim 1 wherein the nucleotide tag sequence is incorporated into the tagged target nucleic acid sequence using a PCR reaction.
3. The method of claim 1 wherein the nucleotide tag recognition sequence is exactly complementary to the nucleotide tag sequence.
4. The method of claim 1 wherein the regulatory oligonucleotide comprises a sequence segment that is exactly complementary to the regulatory sequence.
5. The method of claim 4 wherein the regulatory oligonucleotide has a length in the range of 15-45 nucleotides.
6. The method of claim 1 wherein T_a is in the range of 55-62°C.
7. The method of claim 1 wherein T_a is in the range of 60-64°C.

8. The method of claim 1 wherein T_a is in the range of 60°C-62°C.
9. The method of claim 1 wherein T_{m1} is at least 25°C higher than the annealing temperature (T_a).
10. The method of claim 9 wherein T_{m2} is at least 2°C higher than the annealing temperature (T_a).
11. The method of claim 10 wherein T_{m2} is in the range of 60-75°C.
12. The method of claim 8 wherein the PCR amplification reaction is carried out in a reaction volume greater than 500 nL.
13. The method of claim 12 wherein the PCR amplification reactions are carried out in a multiwell plate comprising 96-1536 wells.
14. The method of claim 8 wherein the PCR amplification reaction is carried out in a reaction volume less than 100 nL.
15. The method of claim 14 wherein the PCR amplification reaction is carried out in a microfluidic device.
16. The method of claim 15 wherein T_a is 60°C.
17. The method of claim 8 wherein the PCR amplification reaction comprises at least 20 cycles at the annealing temperature (T_a).
18. The method of claim 9 wherein T_{m2} is at least 4°C higher than the annealing temperature (T_a).
19. The method of claim 9 wherein T_{m2} is at least 10°C higher than the annealing temperature (T_a).

20. The method of claim 8 wherein the PCR amplification reaction is carried out in a reaction volume greater than 1 uL.

21. A kit comprising:

a) a first tagging primer comprising a first nucleotide tag sequence and a first target nucleotide recognition sequence that hybridizes to a first allele at a polymorphic site in a target nucleic acid sequence;

b) a second tagging primer comprising a second nucleotide tag sequence and a second target nucleotide recognition sequence that hybridizes to a second allele at the polymorphic site in the target nucleic acid sequence;

c) a reverse primer;

d) a first probe oligonucleotide comprising (i) a first nucleotide tag recognition sequence complementary to the first nucleotide tag sequence and (ii) a first regulatory sequence 5' to the first nucleotide tag recognition sequence, wherein the first probe oligonucleotide comprises a first label and has a melting temperature T_{m1} ;

e) a first regulatory oligonucleotide comprising a first sequence segment that is complementary to the first regulatory sequence, wherein the first regulatory oligonucleotide comprises a second label and has a melting temperature T_{m2} ;

f) a second probe oligonucleotide comprising (i) a second nucleotide tag recognition sequence complementary to the second nucleotide tag sequence and (ii) a second regulatory sequence 5' to the second nucleotide tag recognition sequence, wherein the second probe oligonucleotide comprises a third label and has a melting temperature T_{m3} ; and

g) a second regulatory oligonucleotide comprising a second sequence segment that is complementary to the second regulatory sequence, wherein the second regulatory oligonucleotide comprises a fourth label and has a melting temperature T_{m4} ,

wherein T_{m1} , T_{m2} , T_{m3} , and T_{m4} are higher than an annealing temperature T_a of a PCR amplification reaction.

22. A PCR amplification reaction mixture comprising:

a) a first tagged target nucleic acid sequence comprising a first allele at a polymorphic site and a first nucleotide tag sequence;

b) a first probe oligonucleotide comprising (i) a first nucleotide tag recognition sequence complementary to the first nucleotide tag sequence and (ii) a first regulatory sequence 5' to the first

nucleotide tag recognition sequence, wherein the first probe oligonucleotide comprises a first label and has a melting temperature T_{m1} ;

c) a first regulatory oligonucleotide comprising a first sequence segment that is complementary to the first regulatory sequence, wherein the first regulatory oligonucleotide comprises a second label and has a melting temperature T_{m2} ;

d) a second tagged target nucleic acid sequence comprising a second allele at the polymorphic site and a second nucleotide tag sequence;

e) a second probe oligonucleotide comprising (i) a second nucleotide tag recognition sequence complementary to the second nucleotide tag sequence and (ii) a second regulatory sequence 5' to the second nucleotide tag recognition sequence, wherein the second probe oligonucleotide comprises a third label and has a melting temperature T_{m3} ; and

f) a second regulatory oligonucleotide comprising a second sequence segment that is complementary to the second regulatory sequence, wherein the second regulatory oligonucleotide comprises a fourth label and has a melting temperature T_{m4} ,

wherein the PCR amplification reaction mixture possesses an annealing temperature T_a , wherein the first label and the second label constitute a first fluorescent reporter/quencher pair, wherein the third label and the fourth label constitute a second fluorescent reporter/quencher pair, and wherein T_{m1} , T_{m2} , T_{m3} , and T_{m4} are higher than T_a .

23. The kit of claim 21, or the PCR amplification reaction mixture of claim 22, wherein T_a is in the range of 55-62°C.

24. The kit of claim 21, or the PCR amplification reaction mixture of claim 22, wherein T_a is in the range of 60-64°C.

25. The kit of claim 21, or the PCR amplification reaction mixture of claim 22, wherein T_a is in the range of 60°C-62°C.

26. The kit of claim 21, or the PCR amplification reaction mixture of claim 22, wherein T_a is 60°C.

Claims

1. A method for detecting a target nucleotide sequence comprising:
 - a) tagging the target nucleotide sequence with a nucleotide tag sequence, thereby producing a tagged target nucleic acid sequence;
 - b) providing a probe oligonucleotide comprising a nucleotide tag recognition sequence complementary to the nucleotide tag sequence and a regulatory sequence 5' to the nucleotide tag recognition sequence, wherein the regulatory sequence does not hybridize to the nucleotide tag sequence, and wherein said probe oligonucleotide comprises a first label and has a melting temperature T_{m1} ;
 - c) amplifying the tagged target nucleic acid sequence in a PCR amplification reaction using the probe oligonucleotide as a primer, wherein said PCR amplification reaction is characterized by an annealing temperature T_a ;
wherein the PCR amplification reaction is carried out in the presence of a regulatory oligonucleotide comprising a sequence segment that is complementary to the regulatory sequence, wherein the regulatory oligonucleotide does not hybridize to the nucleotide tag recognition sequence, and wherein said regulatory oligonucleotide comprises a second label and has a melting temperature T_{m2} ; and
 - d) detecting the product of the PCR amplification reaction;
wherein the first label and the second label constitute a fluorescent reporter/quencher pair; and
wherein T_{m1} and T_{m2} are both higher than T_a .
2. The method of claim 1 wherein the nucleotide tag sequence is incorporated into the tagged target nucleic acid sequence using a PCR reaction.
3. The method of claim 1 wherein the nucleotide tag recognition sequence is exactly complementary to the nucleotide tag sequence.
4. The method of claim 1 wherein the regulatory oligonucleotide comprises a sequence segment that is exactly complementary to the regulatory sequence.
5. The method of claim 4 wherein the regulatory oligonucleotide has a length in the range of 15-45 nucleotides.
6. The method of claim 1 wherein T_a is in the range of 55-62°C.

7. The method of claim 1 wherein T_a is in the range of 60-64°C.
8. The method of claim 1 wherein T_a is in the range of 60°C-62°C.
9. The method of claim 1 wherein T_{m1} is at least 25°C higher than the annealing temperature (T_a).
10. The method of claim 9 wherein T_{m2} is at least 2°C higher than the annealing temperature (T_a).
11. The method of claim 10 wherein T_{m2} is in the range of 60-75°C.
12. The method of claim 8 wherein the PCR amplification reaction is carried out in a reaction volume greater than 500 nL.
13. The method of claim 12 wherein the PCR amplification reactions are carried out in a multiwell plate comprising 96-1536 wells.
14. The method of claim 8 wherein the PCR amplification reaction is carried out in a reaction volume less than 100 nL.
15. The method of claim 14 wherein the PCR amplification reaction is carried out in a microfluidic device.
16. The method of claim 15 wherein T_a is 60°C.
17. The method of claim 8 wherein the PCR amplification reaction comprises at least 20 cycles at the annealing temperature (T_a).
18. The method of claim 9 wherein T_{m2} is at least 4°C higher than the annealing temperature (T_a).

19. The method of claim 9 wherein T_{m2} is at least 10°C higher than the annealing temperature (T_a).

20. The method of claim 8 wherein the PCR amplification reaction is carried out in a reaction volume greater than 1 uL.

21. A kit comprising:

a) a first tagging primer comprising a first nucleotide tag sequence and a first target nucleotide recognition sequence that hybridizes to a first allele at a polymorphic site in a target nucleic acid sequence;

b) a second tagging primer comprising a second nucleotide tag sequence and a second target nucleotide recognition sequence that hybridizes to a second allele at the polymorphic site in the target nucleic acid sequence;

c) a reverse primer;

d) a first probe oligonucleotide comprising (i) a first nucleotide tag recognition sequence complementary to the first nucleotide tag sequence and (ii) a first regulatory sequence 5' to the first nucleotide tag recognition sequence, wherein the regulatory sequence does not hybridize to the nucleotide tag sequence, wherein the first probe oligonucleotide comprises a first label and has a melting temperature T_{m1} ;

e) a first regulatory oligonucleotide comprising a first sequence segment that is complementary to the first regulatory sequence, wherein the regulatory oligonucleotide does not hybridize to the nucleotide tag recognition sequence, wherein the first regulatory oligonucleotide comprises a second label and has a melting temperature T_{m2} ;

f) a second probe oligonucleotide comprising (i) a second nucleotide tag recognition sequence complementary to the second nucleotide tag sequence and (ii) a second regulatory sequence 5' to the second nucleotide tag recognition sequence, wherein the regulatory sequence does not hybridize to the nucleotide tag sequence, wherein the second probe oligonucleotide comprises a third label and has a melting temperature T_{m3} ; and

g) a second regulatory oligonucleotide comprising a second sequence segment that is complementary to the second regulatory sequence, wherein the regulatory oligonucleotide does not hybridize to the nucleotide tag recognition sequence, wherein the second regulatory oligonucleotide comprises a fourth label and has a melting temperature T_{m4} ,

wherein T_{m1} , T_{m2} , T_{m3} , and T_{m4} are higher than an annealing temperature T_a of a PCR amplification reaction.

22. A PCR amplification reaction mixture comprising:

a) a first tagged target nucleic acid sequence comprising a first allele at a polymorphic site and a first nucleotide tag sequence;

b) a first probe oligonucleotide comprising (i) a first nucleotide tag recognition sequence complementary to the first nucleotide tag sequence and (ii) a first regulatory sequence 5' to the first nucleotide tag recognition sequence, wherein the regulatory sequence does not hybridize to the nucleotide tag sequence, wherein the first probe oligonucleotide comprises a first label and has a melting temperature T_{m1} ;

c) a first regulatory oligonucleotide comprising a first sequence segment that is complementary to the first regulatory sequence, wherein the regulatory oligonucleotide does not hybridize to the nucleotide tag recognition sequence, wherein the first regulatory oligonucleotide comprises a second label and has a melting temperature T_{m2} ;

d) a second tagged target nucleic acid sequence comprising a second allele at the polymorphic site and a second nucleotide tag sequence;

e) a second probe oligonucleotide comprising (i) a second nucleotide tag recognition sequence complementary to the second nucleotide tag sequence and (ii) a second regulatory sequence 5' to the second nucleotide tag recognition sequence, wherein the regulatory sequence does not hybridize to the nucleotide tag sequence, wherein the second probe oligonucleotide comprises a third label and has a melting temperature T_{m3} ; and

f) a second regulatory oligonucleotide comprising a second sequence segment that is complementary to the second regulatory sequence, wherein the regulatory oligonucleotide does not hybridize to the nucleotide tag recognition sequence, wherein the second regulatory oligonucleotide comprises a fourth label and has a melting temperature T_{m4} ,

wherein the PCR amplification reaction mixture possesses an annealing temperature T_a , wherein the first label and the second label constitute a first fluorescent reporter/quencher pair, wherein the third label and the fourth label constitute a second fluorescent reporter/quencher pair, and wherein T_{m1} , T_{m2} , T_{m3} , and T_{m4} are higher than T_a .

23. The kit of claim 21, or the PCR amplification reaction mixture of claim 22, wherein T_a is in the range of 55-62°C.

24. The kit of claim 21, or the PCR amplification reaction mixture of claim 22, wherein T_a is in the range of 60-64°C.

25. The kit of claim 21, or the PCR amplification reaction mixture of claim 22, wherein Ta is in the range of 60°C-62°C.

26. The kit of claim 21, or the PCR amplification reaction mixture of claim 22, wherein Ta is 60°C.

27. The method of claim 1, further comprising detecting a second target nucleotide sequence, wherein the second target nucleotide sequence is different from the target nucleotide sequence of claim 1 by a single nucleotide polymorphism (SNP).

28. The method of claim 27, wherein step a) further comprises tagging the second target nucleotide sequence with a second nucleotide tag sequence, wherein the second nucleotide sequence is different from the nucleotide tag sequence of claim 1.

29. The method of claim 27, further comprising detecting the second target nucleotide sequence using a probe oligonucleotide/regulatory oligonucleotide pair specific for the second nucleotide tag sequence and comprising a second reporter/quencher pair, wherein the second reporter/quencher pair is distinguishable from the reporter/quencher pair of claim 1.