

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 15 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 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 is characterized by 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 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 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 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 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 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 Ta is in the range of 60°C-62°C.
9. The method of claim 1 wherein Tm1 is at least 25°C higher than the annealing temperature (Ta).
10. The method of claim 9 wherein Tm2 is at least 2°C higher than the annealing temperature (Ta).
11. The method of claim 10 wherein Tm2 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 Ta is 60°C.
17. The method of claim 8 wherein the PCR amplification reaction comprises at least 20 cycles at the annealing temperature (Ta).
18. The method of claim 9 wherein Tm2 is at least 4°C higher than the annealing temperature (Ta).
19. The method of claim 9 wherein Tm2 is at least 10°C higher than the annealing temperature (Ta).

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.