

the use of a universal detection system but in principle this can be impacted by the detection of non-specific amplification products.

There is a need for easy-to-synthesise, low cost and reliable, specific detection systems for use in the detection of primer extension products, e.g. in homogeneous PCR assays, which address the problems encountered with existing detection systems for PCR. The term homogeneous PCR assay is well known in the art, and is one where it is not necessary physically to separate the reaction components away from each other in order to derive the result of the reaction. The present invention is based on the finding that selection of the relative lengths of labelled oligonucleotide sequences that hybridise to one another to form a fluorescent quenched pair results in improvements in nucleic acid detection assay systems, particularly when used in a real-time setting. In the invention, the T_m of the fluor/quencher cassette is designed to be above the T_a of the amplification such that any unincorporated fluorescent oligonucleotide is hybridised to the quencher oligonucleotide at the fluorescence acquisition temperature allowing the reaction to be monitored in real-time or at end point. By adjusting the length and T_m of the quencher oligonucleotide it would be expected that the increased stability of the fluor/quencher cassette would simply inhibit PCR. However it is unexpectedly found that the specificity of amplification from the fluorescent primer is improved as shown by significant increases in the difference in C_q values (also known as C_t values) between samples and no template controls in real-time, or reduced detection of no template controls in end point applications.

SUMMARY

In one aspect, there is provided a method for the detection of a primer extension product, the method comprising the steps of:

- a) providing one or more oligonucleotide primer groups, each group comprising one or more oligonucleotide primer sets, each set characterised by
 - i) a first oligonucleotide primer (~~forward primer~~) having a target-specific portion and a 5' upstream fluorescence cassette-specific portion, and
 - ii) a second oligonucleotide primer (~~reverse primer~~) having a target specific portion

wherein the oligonucleotide primers in a particular set are suitable respectively for hybridisation on complementary strands of a corresponding target nucleotide sequence to permit formation of a primer extension product, for example a PCR product

and wherein the first oligonucleotide primer of each set in the same group contains a fluorescence cassette-specific portion that is capable of hybridising to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in the same group

- 5 b) providing one or more cassette oligonucleotide sets, each set characterised by
- i) a first cassette oligonucleotide labelled with a fluorescent moiety that is a (donor moiety) and having a sequence that is capable of hybridisation to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in a given oligonucleotide primer group, and not comprising a target specific sequence portion; and
 - 10 ii) a second cassette oligonucleotide labelled with an acceptor moiety (for example a quencher moiety), wherein the second cassette oligonucleotide does not comprise target specific sequence and wherein the second cassette oligonucleotide is between 1 and 5 nucleotides shorter than the corresponding first cassette oligonucleotide;
- wherein each set of cassette oligonucleotides hybridises to one another to form a fluorescent
- 15 quenched pair, wherein the fluorescent quenched pair has a $T_m A$,
- c) initiating the primer extension reaction thereby generating (if the relevant target polynucleotide is present) a complementary sequence to the relevant first oligonucleotide primer,
- 20 such that the relevant second ~~(acceptor, for example quencher, labelled)~~ cassette oligonucleotide is less able to hybridise to the relevant first ~~(fluorescently labelled)~~ cassette oligonucleotide, whereby a signal is generated; and
- d) detecting the signal that is generated,

wherein the primer extension reaction is performed at least in part at a T_a that is less than

25 the $T_m A$ or $T_m A_s$ for the one or more fluorescent quenched pairs.

Kits suitable for use in such a method are also provided.

In one aspect, there is provided a kit suitable for use in a method for the detection of a

30 primer extension product, ~~the method comprising the steps of~~ kit comprising:

- a) two or more oligonucleotide primer groups, each group comprising one or more oligonucleotide primer sets, each set characterised by
 - i) a first oligonucleotide primer ~~(forward primer)~~ having a target-specific portion and a 5' upstream fluorescence cassette-specific portion; and
 - 35 ii) a second oligonucleotide primer ~~(reverse primer)~~ having a target specific portion

wherein the oligonucleotide primers in a particular set are suitable respectively for hybridisation on complementary strands of a corresponding target nucleotide sequence to permit formation of a primer extension product, for example a PCR product

and wherein the first oligonucleotide primer of each set in the same group contains a fluorescence cassette-specific portion that is capable of hybridising to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in the same group ; and

b) two or more cassette oligonucleotide sets, each set characterised by

i) a first cassette oligonucleotide labelled with a fluorescent moiety that is a (donor moiety) and having a sequence that is capable of hybridisation to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in a given oligonucleotide primer group and not comprising a target specific sequence portion; and

ii) a second cassette oligonucleotide labelled with an acceptor moiety (for example a quencher moiety), wherein the second cassette oligonucleotide does not comprise target specific sequence and wherein the second cassette oligonucleotide is between 1 and 5 nucleotide shorter than the corresponding first cassette oligonucleotide;

wherein each set of cassette oligonucleotides hybridises to one another to form a fluorescent quenched pair, wherein the fluorescent quenched pair has a T_m A,

wherein each of the T_m As for the fluorescent quenched pairs is above a temperature suitable for use as the T_a of a primer extension reaction using the oligonucleotides of the kit, for example above a temperature between 46 and 65 °C, for example between 50 and 60 °C.

In one aspect embodiment, there is provided a method for the detection of a primer extension product, the method comprising the steps of:

a) providing one or more oligonucleotide primer groups, each group comprising one or more oligonucleotide primer sets, each set characterised by

i) a first labelled oligonucleotide primer (forward primer) having a target-specific portion and a 5' upstream fluorescence cassette-specific portion, and

ii) a second oligonucleotide primer (reverse primer) having a target specific portion

wherein the oligonucleotide primers in a particular set are suitable respectively for hybridisation on complementary strands of a corresponding target nucleotide sequence to permit formation of a primer extension product, for example a PCR product

and wherein the first oligonucleotide primer of each set in the same group contains a fluorescence cassette-specific portion that is capable of hybridising to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in the same group

CLAIMS

1. A method for the detection of a primer extension product, the method comprising the steps of:

- a) providing one or more oligonucleotide primer groups, each group comprising one or more oligonucleotide primer sets, each set characterised by
 - i) a first oligonucleotide primer having a target-specific portion and a 5' upstream fluorescence cassette-specific portion, and
 - ii) a second oligonucleotide primer having a target specific portion

wherein the oligonucleotide primers in a particular set are suitable respectively for hybridisation on complementary strands of a corresponding target nucleotide sequence to permit formation of a primer extension product, for example a PCR product

and wherein the first oligonucleotide primer of each set in the same group contains a fluorescence cassette-specific portion that is capable of hybridising to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in the same group

- b) providing one or more cassette oligonucleotide sets, each set characterised by
 - i) a first cassette oligonucleotide labelled with a fluorescent moiety that is a donor moiety and having a sequence that is capable of hybridisation to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in a given oligonucleotide primer group, and not comprising a target specific sequence portion; and
 - ii) a second cassette oligonucleotide labelled with an acceptor moiety (for example a quencher moiety), wherein the second cassette oligonucleotide does not comprise target specific sequence and wherein the second cassette oligonucleotide is between 1 and 5 nucleotide bases shorter than the corresponding first cassette oligonucleotide;

wherein each set of cassette oligonucleotides hybridises to one another to form a fluorescent quenched pair, wherein the fluorescent quenched pair has a T_m A,

- c) initiating the primer extension reaction thereby generating (if the relevant target polynucleotide is present) a complementary sequence to the relevant first oligonucleotide primer,

such that the relevant second cassette oligonucleotide is less able to hybridise to the relevant first cassette oligonucleotide, whereby a signal is generated; and

d) detecting the signal that is generated,

wherein the primer extension reaction is performed at least in part at a T_a that is less than the T_m A or T_m As for the one or more fluorescent quenched pairs.

2. The method of claim 1 wherein the T_m A of the fluorescent quenched pair or pairs is less than or equal to 15°C, optionally between 1 and 15°C; or less than or equal to 10°C, optionally between 1 and 10°C above the T_a of the primer extension reaction.

3. A method according to claim 1 or 2 wherein the method prevents or reduces detection of non-specific amplification.

4. A method according to any one of the preceding claims wherein the signal is measured in real-time.

5. A method according to any one of claim 1, 2 or 3 wherein the signal is measured at the end point of the reaction.

6. The method of any one of claims 1 to 5 wherein the or a first cassette oligonucleotide labelled with a fluorescent moiety is capable of acting as a primer in a primer extension reaction.

7. The method of any one of claims 1 to 6 wherein the or a first cassette oligonucleotide labelled with a fluorescent moiety is not capable of acting as a primer in the primer extension reaction.

8. The method of any one of claims 1 to 7 wherein the interaction between the fluorescent donor labelled fluorescence cassette oligonucleotide and the acceptor labelled fluorescence cassette oligonucleotide is less stable than the interaction between the fluorescent donor labelled fluorescence cassette oligonucleotide and the extension product complementary to the 5' upstream fluorescence cassette-specific portion of the first oligonucleotide primer of each primer set of the relevant group.

9. The method of any one of the preceding claims wherein each oligonucleotide primer group comprises one oligonucleotide primer set.

10. The method of any one of the preceding claims wherein there are 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 oligonucleotide primer groups and corresponding cassette oligonucleotide sets.

11. The method of any one of the preceding claims wherein one or both of the cassette oligonucleotides contains a single label.

12. The method of any one of the preceding claims wherein both of the cassette oligonucleotides contain a single label.

13. The method according to any one of the preceding claims wherein the fluorescent labelled cassette oligonucleotide contains a label at or within the 5' end of the oligonucleotide.

14. The method according to any one of the preceding claims wherein the quencher labelled cassette oligonucleotide contains a label at or within the 3' end of the oligonucleotide.

15. The method according to any one of the preceding claims wherein at least one of the bases of at least one of the oligonucleotides, for example at least one of the cassette oligonucleotides, optionally the fluorescent labelled cassette oligonucleotide, is a phosphorothioate-modified base.

16. The method according to claim 15, wherein 20-80% of the bases of at least one of the oligonucleotides, for example at least one of the cassette oligonucleotides, optionally the fluorescent labelled cassette oligonucleotide, are phosphorothioate-modified bases.

17. A kit suitable for use in a method for the detection of a primer extension product, the kit comprising:

- a) two or more oligonucleotide primer groups, each group comprising one or more oligonucleotide primer sets, each set characterised by
 - i) a first oligonucleotide primer having a target-specific portion and a 5' upstream fluorescence cassette-specific portion, and
 - ii) a second oligonucleotide primer having a target specific portion

wherein the oligonucleotide primers in a particular set are suitable respectively for hybridisation on complementary strands of a corresponding target nucleotide sequence to permit formation of a primer extension product, for example a PCR product and wherein the first oligonucleotide primer of each set in the same group contains a fluorescence cassette-specific portion that is capable of hybridising to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in the same group ; and

b) two or more cassette oligonucleotide sets, each set characterised by

i) a first cassette oligonucleotide labelled with a fluorescent moiety that is a donor moiety and having a sequence that is capable of hybridisation to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in a given oligonucleotide primer group and not comprising a target specific sequence portion; and

ii) a second cassette oligonucleotide labelled with an acceptor moiety (for example a quencher moiety), wherein the second cassette oligonucleotide does not comprise target specific sequence and wherein the second cassette oligonucleotide is between 1 and 5 nucleotide bases shorter than the corresponding first cassette oligonucleotide;

wherein each set of cassette oligonucleotides hybridises to one another to form a fluorescent quenched pair, wherein the fluorescent quenched pair has a T_m A,

wherein each of the T_m As for the fluorescent quenched pairs is above a temperature suitable for use as the T_a of a primer extension reaction using the oligonucleotides of the kit, for example above a temperature between 46 and 65 °C, for example between 50 and 60 °C.

18. The kit of claim 17 wherein the first oligonucleotide primers are unlabelled.

19. The method or kit according to any one of the preceding claims, for use in allele specific PCR based SNP Genotyping, gene expression studies or copy number variation studies.

CLAIMS

1. A method for the detection of a primer extension product, the method comprising the steps of:

- 5 a) providing one or more oligonucleotide primer groups, each group comprising one or more oligonucleotide primer sets, each set characterised by
- i) a first oligonucleotide primer (forward primer) having a target-specific portion and a 5' upstream fluorescence cassette-specific portion, and
- 10 ii) a second oligonucleotide primer (reverse primer) having a target specific portion

wherein the oligonucleotide primers in a particular set are suitable respectively for hybridisation on complementary strands of a corresponding target nucleotide sequence to permit formation of a primer extension product, for example a PCR product

15 and wherein the first oligonucleotide primer of each set in the same group contains a fluorescence cassette-specific portion that is capable of hybridising to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in the same group

- b) providing one or more cassette oligonucleotide sets, each set characterised by

20 i) a first cassette oligonucleotide labelled with a fluorescent moiety (donor moiety) and having a sequence that is capable of hybridisation to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in a given oligonucleotide primer group; and

25 ii) a second cassette oligonucleotide labelled with an acceptor moiety (for example a quencher moiety)

wherein each set of cassette oligonucleotides hybridises to one another to form a fluorescent quenched pair, wherein the fluorescent quenched pair has a $T_m A$,

- c) initiating the primer extension reaction thereby generating (if the relevant target polynucleotide is present) a complementary sequence to the relevant first oligonucleotide primer,

30 such that the relevant second (acceptor, for example quencher, labelled) cassette oligonucleotide is less able to hybridise to the relevant first (fluorescently labelled) cassette oligonucleotide, whereby a signal is generated; and

- d) detecting the signal that is generated,

wherein the primer extension reaction is performed at least in part at a T_a that is less than the T_m A or T_m As for the one or more fluorescent quenched pairs.

2. The method of claim 1 wherein the T_m (T_m A) of the fluorescent quenched pair or pairs is less than or equal to 15°C , optionally between 1 and 15°C ; or less than or equal to 10°C , optionally between 1 and 10°C above the T_a of the primer extension reaction.

3. A method according to claim 1 or 2 wherein the method prevents or reduces detection of non-specific amplification.

4. A method according to any one of the preceding claims wherein the signal is measured in real-time.

5. A method according to any one of claim 1, 2 or 3 wherein the signal is measured at the end point of the reaction.

6. The method of any one of claims 1 to 5 wherein the or a first cassette oligonucleotide labelled with a fluorescent moiety is capable of acting as a primer in a primer extension reaction.

7. The method of any one of claims 1 to 6 wherein the or a first cassette oligonucleotide labelled with a fluorescent moiety is not capable of acting as a primer in the primer extension reaction.

8. The method of any one of claims 1 to 7 wherein the interaction between the fluorescent (donor) labelled fluorescence cassette oligonucleotide and the acceptor (quencher) labelled fluorescence cassette oligonucleotide is less stable than the interaction between the fluorescent (donor) labelled fluorescence cassette oligonucleotide and the extension product complementary to the 5' upstream fluorescence cassette-specific portion of the forward oligonucleotide primer of each primer set of the relevant group.

9. The method of any one of claims 1 to 8 wherein the acceptor labelled oligonucleotide is between 1 and 5 nucleotide bases shorter than the corresponding first (fluorescently labelled) cassette oligonucleotide.

10. The method of any one of the preceding claims wherein each oligonucleotide primer group comprises one oligonucleotide primer set.

5

11. The method of any one of the preceding claims wherein there are 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 oligonucleotide primer groups and corresponding cassette oligonucleotide sets.

12. The method of any one of the preceding claims wherein one or both of the cassette oligonucleotides contains a single label.

10

13. The method of claim 12 wherein both of the cassette oligonucleotides contain a single label.

14. The method according to any one of the preceding claims wherein the fluorescent labelled cassette oligonucleotide contains a label at or within the 5' end of the oligonucleotide.

15

15. The method or kit according to any one of the preceding claims wherein the quencher labelled cassette oligonucleotide contains a label at or within the 3' end of the oligonucleotide.

20

16. The method according to any one of the preceding claims wherein at least one of the bases of at least one of the oligonucleotides, for example at least one of the cassette oligonucleotides, optionally the fluorescent labelled cassette oligonucleotide, is a phosphorothioate.

25

17. The method according to claim 16 wherein 20-80% of the bases of at least one of the oligonucleotides, for example at least one of the cassette oligonucleotides, optionally the fluorescent labelled cassette oligonucleotide, are phosphorothioates.

30

18. A kit suitable for use in a method for the detection of a primer extension product, the method comprising the steps of:

a) two or more oligonucleotide primer groups, each group comprising one or more oligonucleotide primer sets, each set characterised by

35

- i) a first oligonucleotide primer (forward primer) having a target-specific portion and a 5' upstream fluorescence cassette-specific portion, and
- ii) a second oligonucleotide primer (reverse primer) having a target specific portion

wherein the oligonucleotide primers in a particular set are suitable respectively for hybridisation on complementary strands of a corresponding target nucleotide sequence to permit formation of a primer extension product, for example a PCR product

and wherein the first oligonucleotide primer of each set in the same group contains a fluorescence cassette-specific portion that is capable of hybridising to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in the same group ; and

b) two or more cassette oligonucleotide sets, each set characterised by

- i) a first cassette oligonucleotide labelled with a fluorescent moiety (donor moiety) and having a sequence that is capable of hybridisation to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in a given oligonucleotide primer group; and

- ii) a second cassette oligonucleotide labelled with an acceptor moiety (for example a quencher moiety)

wherein each set of cassette oligonucleotides hybridises to one another to form a fluorescent quenched pair, wherein the fluorescent quenched pair has a $T_m A$,

wherein each of the $T_m A$ s for the fluorescent quenched pairs is above a temperature suitable for use as the T_a of a primer extension reaction using the oligonucleotides of the kit, for example above a temperature between 46 and 65 °C, for example between 50 and 60 °C.

19. The kit of claim 18 wherein the first oligonucleotide primers are unlabelled and wherein the quencher labelled cassette oligonucleotide is between 1 and 5 nucleotide bases shorter than the fluorescent labelled cassette oligonucleotide.

20. A method for the detection of a primer extension product, the method comprising the steps of:

- a) providing one or more oligonucleotide primer groups, each group comprising one or more oligonucleotide primer sets, each set characterised by

- i) a first labelled oligonucleotide primer (forward primer) having a target-specific portion and a 5' upstream fluorescence cassette-specific portion, and

- ii) a second oligonucleotide primer (reverse primer) having a target specific portion

wherein the oligonucleotide primers in a particular set are suitable respectively for hybridisation on complementary strands of a corresponding target nucleotide sequence to permit formation of a primer extension product, for example a PCR product

and wherein the first oligonucleotide primer of each set in the same group contains a fluorescence cassette-specific portion that is capable of hybridising to the complement of the fluorescence cassette-specific portion of the first oligonucleotide primer of any set in the same group

b) providing one or more cassette oligonucleotide sets, each set characterised by

i) a first cassette oligonucleotide or oligonucleotides labelled with a fluorescent moiety (donor moiety) that is the first labelled oligonucleotide primer or primers (forward primer or primers) of a primer group

ii) a second cassette oligonucleotide labelled with an acceptor moiety (for example a quencher moiety)

wherein each set of cassette oligonucleotides hybridises to one another to form a fluorescent quenched pair, wherein the fluorescent quenched pair has a $T_m A$,

c) initiating the primer extension reaction thereby generating (if the relevant target polynucleotide is present) a complementary sequence to the relevant first oligonucleotide primer,

such that the relevant second (acceptor, for example quencher, labelled) cassette oligonucleotide is less able to hybridise to the relevant first (fluorescently labelled) cassette oligonucleotide, whereby a signal is generated; and

d) detecting the signal that is generated,

wherein the primer extension reaction is performed at least in part at a T_a that is less than the $T_m A$ or $T_m A_s$ for the one or more fluorescent quenched pairs.

21. The method or kit according to any one of the preceding claims, for use in allele specific PCR based SNP Genotyping, gene expression studies or copy number variation studies.