

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 OF THE INVENTION

~~According to the invention~~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

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b) providing one 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

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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 $T_m A$,

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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

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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.

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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 primer extension product, the method comprising the steps of:

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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

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

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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.

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 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)
5 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 $T_m A$,
10 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
15 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.

20 In one aspect, there is provided the method or kit as defined herein, for use in allele specific
PCR based SNP Genotyping, gene expression studies or copy number variation studies.

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

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a simple reaction schema for detection of a DNA sequence in SNP Genotyping
30 embodying a method of the present invention.

Figure 2 shows data generated using the assay described in Example 2 below. Example
genotyping data for amplification products of fluor/quencher Cassette 5 (left) and
fluor/quencher Cassette 1 (right). The three clusters present in each example represent the