

CLAIMS

1. A method for the detection of a primer extension product produced in the presence of a polymerase lacking 3'→5' exonuclease activity, the method comprising the steps of:
- 5 a) providing at least two single-labelled oligonucleotide sequences that hybridise to one another in free solution to form a fluorescent quenched pair, that upon introduction of a complementary sequence to one or both sequences generates a measurable signal when the complementary sequence is hybridised to one of the at least two single-labelled oligonucleotide sequences, wherein at least one of the oligonucleotide sequences contains at least one phosphorothioate group;
- 10 b) providing at least one primer and initiating the primer extension reaction from the at least one primer thereby generating a complementary sequence to at least one of the single-labelled oligonucleotide sequences; and
- 15 c) measuring the detectable signal that is generated when the complementary sequence is hybridised to one of the at least two single-labelled oligonucleotide sequences.
2. A kit for the detection of a primer extension product produced in the presence of a polymerase lacking 3'→5' exonuclease activity, that comprises at least two single-labelled oligonucleotide sequences that hybridise to one another in free solution to form a fluorescent quenched pair, that upon introduction of a complementary sequence to one or both sequences generates a measurable signal when the complementary sequence is hybridised to one of the two single-labelled oligonucleotide sequences, wherein at least one of the oligonucleotide sequences contains at least one phosphorothioate group and wherein:
- 20 at least one of the internal bases of the at least one oligonucleotide sequence contains a phosphorothioate group, and;
- 25
- 30 wherein the modified bases are separated by at least one unmodified base, and/or
wherein alternate bases are phosphorothioates, and/or
wherein at least three of the bases are phosphorothioates, and/or
- 35 wherein the 5' terminus does not contain a phosphorothioate group.
3. The method or kit according to claim 1 or 2, wherein the first and second oligonucleotide sequences are of different T_m (melting temperature)
- 40 4. The method or kit according to claim 3, wherein one of the first and second oligonucleotide sequences has a T_m that is at or below the T_a (annealing temperature) of the primer extension reaction.

5. The method or kit according to claim 3 or 4, wherein one of the first and second oligonucleotide sequences has a T_m that is above the T_a of the primer extension reaction.

5 6. A method for the detection of a primer extension product produced in the presence of a polymerase lacking 3'→5' exonuclease activity using PCR, the method comprising the steps of:

10 a) providing a first single-labelled oligonucleotide sequence and at least a second single-labelled oligonucleotide sequence, the first and second oligonucleotide sequences being of differing T_m , in which the first and second oligonucleotide sequences hybridise to one another in free solution to form a fluorescent quenched pair and at least one primer, one of the first and second oligonucleotide sequences being of a T_m that is at or below the T_a of the PCR process, wherein at least one of the oligonucleotide sequences contains at least one phosphorothioate group, the at least one primer comprising at least one unlabelled
15 tailed primer, the unlabelled tailed primer having a tail region, the tail region comprising an oligonucleotide sequence complementary to an oligonucleotide sequence of the second single-labelled oligonucleotide sequence, the first single-labelled oligonucleotide sequence being a primer from which DNA synthesis is initiated once a complementary sequence to the first single-labelled oligonucleotide
20 sequence has been generated during the PCR process, such that the second single-labelled oligonucleotide sequence is no longer able to hybridise to the first single-labelled oligonucleotide sequence, whereby a measurable signal is generated;

25 b) initiating the primer extension reaction from the at least one primer thereby generating a complementary sequence to the first single-labelled oligonucleotide sequence; and

30 c) measuring the detectable signal that is generated when the complementary sequence is hybridised to the first single-labelled oligonucleotide sequence.

7. A method or kit according to any one of claims 1 to 6, wherein one of the single-labelled oligonucleotides is more than 10 bases shorter than the other.

35 8. A method according to any one of claims 1 and 3 to 5 wherein the PCR process is monitored in real time at each cycle or after a number of cycles where the reaction has otherwise not yet generated enough product to create a measurable signal by lowering the temperature of the reaction to allow hybridisation to occur.

40 9. A method according to any one of claims 6 to 8, wherein said other of the single-labelled oligonucleotides has a T_m that is above the T_a .

10. A method or kit according to any one of claims 1 to 9, wherein said one of the single-labelled oligonucleotides has the quencher label of the fluorescent
45 quenched pair.

11. A method according to any one of the preceding claims wherein at least one of the internal bases of the phosphorothioate group containing oligonucleotide(s) is a phosphorothioate, and/or wherein 20-80% of the bases of phosphorothioate group containing oligonucleotide(s) are phosphorothioates, and/or
5 wherein the modified bases are separated by at least one unmodified base, and/or
wherein alternate bases are phosphorothioates, and/or
wherein at least three of the bases are phosphorothioates, and/or
10 wherein the 5' terminus does not contain a phosphorothioate group.
12. A method or kit according to any one of the preceding claims, for use in allele specific PCR based SNP Genotyping.
- 15 13. A method or kit according to any one of the preceding claims, for monitoring the production of an amplicon via the 5' nuclease assay, wherein the 5' nuclease assay is being employed to perform allelic discrimination reactions.
- 20 14. A method according to any one of claims 1, 3 to 13, wherein the primer extension product is monitored via the use of hybridisation only, optionally wherein the primer extension product is monitored via the use of hybridisation only post PCR.
- 25 15. A method or kit according to any one of claims 1 to 14, wherein the fluorescent quench oligo pairs range from 6 bp to 100 bp, optionally range from 6 bp to 100 bp but are not matched in length.
- 30 16. A method or a kit according to any one of the preceding claims wherein at least one of the bases of a fluorophore-labelled oligonucleotide contains at least one phosphorothioate group.
17. A method or kit according to any one of the preceding claims, wherein the fluorescent quench oligo pairs are:
- 35 a) labelled one of the pair with a fluorophore and the other with a non fluorescent quenching molecule; and/or
b) modified to be resistant to nuclease degradation; and/or
40 are labelled with molecules that are distance sensitive.

CLAIMS

1. A method for the detection of a primer extension product produced in the presence of a polymerase lacking 3'→5' exonuclease activity, the method comprising the steps of:

- a) providing at least two single-labelled oligonucleotide sequences that hybridise to one another in free solution to form a fluorescent quenched pair, that upon introduction of a complementary sequence to one or both sequences generates a measurable signal when the complementary sequence is hybridised to one of the at least two single-labelled oligonucleotide sequences, wherein at least one of the oligonucleotide sequences contains at least one phosphorothioate group;
- b) providing at least one primer and initiating the primer extension reaction from the at least one primer thereby generating a complementary sequence to at least one of the single-labelled oligonucleotide sequences; and
- c) measuring the detectable signal that is generated when the complementary sequence is hybridised to one of the at least two single-labelled oligonucleotide sequences.

2. A kit for the detection of a primer extension product produced in the presence of a polymerase lacking 3'→5' exonuclease activity, that comprises at least two single-labelled oligonucleotide sequences that hybridise to one another in free solution to form a fluorescent quenched pair, that upon introduction of a complementary sequence to one or both sequences generates a measurable signal when the complementary sequence is hybridised to one of the two single-labelled oligonucleotide sequences, wherein at least one of the oligonucleotide sequences contains at least one phosphorothioate group and wherein:

at least one of the internal bases of the at least one oligonucleotide sequence contains a phosphorothioate group, and;

- wherein the modified bases are separated by at least one unmodified base, and/or
- wherein alternate bases are phosphorothioates, and/or
- wherein at least three of the bases are phosphorothioates, and/or
- wherein the 5' terminus does not contain a phosphorothioate group.

3. The method or kit according to claim 1 or 2, wherein the first and second oligonucleotide sequences are of different T_m (melting temperature), optionally

- a) wherein one of the first and second oligonucleotide sequences has a T_m that is at or below the T_a (annealing temperature) of the primer extension reaction, or

- b) wherein one of the first and second oligonucleotide sequences has a T_m that is above the T_a of the primer extension reaction.

4. A method for the detection of a primer extension product produced in the presence of a polymerase lacking 3'→5' exonuclease activity using PCR, the method comprising the steps of:

- 5 a) providing a first single-labelled oligonucleotide sequence and at least a second single-labelled oligonucleotide sequence, the first and second oligonucleotide sequences being of differing T_m , in which the first and second oligonucleotide sequences hybridise to one another in free solution to form a fluorescent quenched pair and at least one primer, one of the first and second
- 10 oligonucleotide sequences being of a T_m that is at or below the T_a of the PCR process, wherein at least one of the oligonucleotide sequences contains at least one phosphorothioate group, the at least one primer comprising at least one unlabelled tailed primer, the unlabelled tailed primer having a tail region, the tail region comprising an oligonucleotide sequence complementary to an oligonucleotide
- 15 sequence of the second single-labelled oligonucleotide sequence, the first single-labelled oligonucleotide sequence being a primer from which DNA synthesis is initiated once a complementary sequence to the first single-labelled oligonucleotide sequence has been generated during the PCR process, such that the second single-labelled oligonucleotide sequence is no longer able to hybridise to the first
- 20 single-labelled oligonucleotide sequence, whereby a measurable signal is generated;
- b) initiating the primer extension reaction from the at least one primer thereby generating a complementary sequence to the first single-labelled oligonucleotide sequence; and
- 25 c) measuring the detectable signal that is generated when the complementary sequence is hybridised to the first single-labelled oligonucleotide sequence.

5. A method or kit according to any one of claims 1 to 4, wherein one of the

30 single-labelled oligonucleotides is more than 10 bases shorter than the other.

6. A method according to any one of claims 1 and 3 wherein the PCR process is monitored in real time at each cycle or after a number of cycles where the reaction has otherwise not yet generated enough product to create a measurable signal by

35 lowering the temperature of the reaction to allow hybridisation to occur.

7. A method according to any one of claims 4 to 6, wherein said other of the single-labelled oligonucleotides has a T_m that is above the T_a .

40 8. A method or kit according to any one of claims 1 to 7, wherein said one of the single-labelled oligonucleotides has the quencher label of the fluorescent quenched pair.

9. A method according to any one of the preceding claims wherein at least one of the internal bases of the phosphorothioate group containing oligonucleotide(s) is a phosphorothioate, and/or wherein 20-80% of the bases of phosphorothioate group containing oligonucleotide(s) are phosphorothioates, and/or
- 5 wherein the modified bases are separated by at least one unmodified base, and/or
- wherein alternate bases are phosphorothioates, and/or
- wherein at least three of the bases are phosphorothioates, and/or
- wherein the 5' terminus does not contain a phosphorothioate group.
- 10
10. A method or kit according to any one of the preceding claims, for use in allele specific PCR based SNP Genotyping.
11. A method or kit according to any one of the preceding claims, for monitoring the production of an amplicon via the 5' nuclease assay, wherein the 5' nuclease assay is being employed to perform allelic discrimination reactions.
- 15
12. A method according to any one of claims 1, 3 to 11, wherein the primer extension product is monitored via the use of hybridisation only, optionally wherein the primer extension product is monitored via the use of hybridisation only post PCR.
- 20
13. A method or kit according to any one of claims 1 to 12, wherein the fluorescent quench oligo pairs range from 6 bp to 100 bp, optionally range from 6 bp to 100 bp but are not matched in length.
- 25
14. A method or a kit according to any one of the preceding claims wherein at least one of the bases of a fluorophore-labelled oligonucleotide contains at least one phosphorothioate group.
- 30
15. A method or kit according to any one of the preceding claims, wherein the fluorescent quench oligo pairs are:
- 35 a) labelled one of the pair with a fluorophore and the other with a non fluorescent quenching molecule; and/or
- b) modified to be resistant to nuclease degradation; and/or
- are labelled with molecules that are distance sensitive.
- 40

The kits according to the invention may also contain a polymerase lacking 3'→5' exonuclease activity and/or other components suitable for use in primer extension reactions such as magnesium salts, dNTPs etc.

- 5 In the present invention, all or at least one of primers used in the methods may contain phosphorothioate-modified bases. The number of phosphodiester linkages replaced by phosphorothioates in any given primer can range from one to all of the phosphodiester bonds being replaced by phosphorothioates. The primer(s) may contain phosphorothioates at the 5' and/or 3' terminii, however it is preferred that, as an alternative to or addition to such terminal modifications, at least one of the internal bases of the primer is a
10 phosphorothioate. For example 10-90%, 20-80%, 30-70% or 40-60% of the bases may be phosphorothioates. In one embodiment the phosphorothioate-modified bases are separated by at least one, e.g. one to three, unmodified (phosphodiester) bases. In a preferred embodiment alternate bases within the primer(s) are phosphorothioates.
- 15 Use of phosphorothioate modification on alternate bases of fluorophore-labelled primers (referred to herein as semi-S modification) in conjunction with unmodified quenchers represents a preferred embodiment of the invention as these give particularly enhanced discrimination and signal intensity of the PCR.

20 Examples of differing phosphorothioate-modifications which may be used in the invention are illustrated below in primers labelled with fluorophores or quenchers, where *denotes a phosphorothioate:

- | | |
|------------------------------|---|
| Unmodified FAM Fluor: | 5'-FAM- GCGATTAGCCGTTAGGATGA 3' (SEQ ID NO: 1) |
| 3'S FAM Fluor: | 5'-FAM- GCGATTAGCCGTTAGGATG*A 3' (SEQ ID NO: 2) |
| Semi S FAM Fluor: | 5'-FAM- G*CG*AT*TA*GC*CG*TT*AG*GA*TG*A 3' (SEQ ID NO: 3) |
| 25 Full S FAM Fluor: | 5'-FAM- G*C*G*A*T*T*A*G*C*C*G*T*T*A*G*G*A*T*G*A 3' (SEQ ID NO: 4) |
| Unmodified HEX Fluor: | 5'-HEX- GTCGGTGAACAGGTTAGAGA 3' (SEQ ID NO: 5) |
| 3' S HEX Fluor: | 5'-HEX- GTCGGTGAACAGGTTAGAG*A 3' (SEQ ID NO: 6) |
| 30 Semi S HEX Fluor: | 5'-HEX- G*TC*GG*TG*AA*CA*GG*TT*AG*AG*A 3' (SEQ ID NO: 7) |
| Full S HEX Fluor: | 5'-HEX- G*T*C*G*G*T*G*A*A*C*A*G*G*T*T*A*G*A*G*A 3' (SEQ ID NO: 8) |
| Standard FAM Quencher: | 5' CCTAACGGCTAATCGC -3'Dabsyl (SEQ ID NO: 9) |
| 35 Semi S FAM Quencher V1.0: | 5' C*CT*AA*CG*GC*TA*AT*CG*C -3'Dabsyl (SEQ ID NO: 10) |
| Semi S FAM Quencher V2.0: | 5' CC*TA*AC*GG*CT*AA*TC*GC -3'Dabsyl (SEQ ID NO: 11) |
| Full S FAM Quencher: | 5' C*C*T*A*A*C*G*G*C*T*A*A*T*C*G*C -3'Dabsyl (SEQ ID NO: 12) |
| Standard HEX Quencher: | 5' AACCTGTTACCGAC-3'Dabsyl (SEQ ID NO: 13) |
| 40 Semi S HEX Quencher V1.0: | 5' AA*CC*TG*TT*CA*CC*GA*C-3'Dabsyl (SEQ ID NO: 14) |
| Semi S HEX Quencher V2.0: | 5' A*AC*CT*GT*TC*AC*CG*AC-3'Dabsyl (SEQ ID NO: 15) |
| Full S HEX Quencher: | 5' A*A*C*C*T*G*T*T*C*A*C*C*G*A*C-3'Dabsyl (SEQ ID NO: 16) |

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range, and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

All publications mentioned herein are incorporated herein by reference to the fullest extent possible for the purpose of describing and disclosing those components that are described in the publications which might be used in connection with the presently described invention.

The invention will now be described by reference to the following examples which are for illustrative purposes and are not to be construed as a limitation of the scope of the present invention.

EXAMPLE 1

Abbreviations:

FAM: 6-Carboxy Fluorescein

HEX: 2',4',5',7',1,4-hexachlorofluorescein

Dabcyl : non-fluorescent dark quencher

Seven oligonucleotides were designed and their sequences can be found below, where *denotes use of S-modification:

1) Semi S FAM fluorescently-labelled oligonucleotide:

5'-FAM-G*CG*AT*TA*GC*CG*TT*AG*GA*TG*A3' (SEQ ID NO: 3)

2) Semi S HEX fluorescently-labelled oligonucleotide: 5'-HEX-

G*TC*GG*TG*AA*CA*GG*TT*AG*AG*A3' (SEQ ID NO: 7)

3) Standard FAM Quencher: 5' CCTAACGGCTAATCGC -3'Dabsyl (SEQ ID NO: 9)

4) Standard HEX Quencher: 5'AACCTGTTCACCGAC-3'Dabsyl (SEQ ID NO: 13)

5) Allele specific primer 1:

5'GCGATTAGCCGTTAGGATGACTGAGTGCAGGTTTCAGACGTCC3' (SEQ ID NO: 17)

6) Allele specific primer 2:

5'GTCGGTGAACAGGTTAGAGACTGAGTGCAGGTTTCAGACGTCT3' (SEQ ID NO: 18)

7) Common reverse primer: 5' CTCCTTCCACCTCCGTACCAT3' (SEQ ID NO: 19)

It will be noted that in this example the FAM-labelled quenching primer has a 16mer oligonucleotide sequence and is more than 2 nucleotides shorter than the FAM-labelled primer/reporter probe and similarly the HEX-labelled quenching primer has a 15mer oligonucleotide sequence and is more than

The kits according to the invention may also contain a polymerase lacking 3'→5' exonuclease activity and/or other components suitable for use in primer extension reactions such as magnesium salts, dNTPs etc.

- 5 In the present invention, all or at least one of primers used in the methods may contain phosphorothioate-modified bases. The number of phosphodiester linkages replaced by phosphorothioates in any given primer can range from one to all of the phosphodiester bonds being replaced by phosphorothioates. The primer(s) may contain phosphorothioates at the 5' and/or 3' termini, however it is preferred that, as an alternative to or addition to such terminal modifications, at least one of the internal bases of the primer is a phosphorothioate. For example 10-90%, 20-80%, 30-70% or 40-60% of the bases may be phosphorothioates. In one embodiment the phosphorothioate-modified bases are separated by at least one, e.g. one to three, unmodified (phosphodiester) bases. In a preferred embodiment alternate bases within the primer(s) are phosphorothioates.
- 15 Use of phosphorothioate modification on alternate bases of fluorophore-labelled primers (referred to herein as semi-S modification) in conjunction with unmodified quenchers represents a preferred embodiment of the invention as these give particularly enhanced discrimination and signal intensity of the PCR.
- 20 Examples of differing phosphorothioate-modifications which may be used in the invention are illustrated below in primers labelled with fluorophores or quenchers, where *denotes a phosphorothioate:

- | | |
|------------------------------|--|
| Unmodified FAM Fluor: | 5'-FAM- GCGATTAGCCGTTAGGATGA 3' |
| 25 3'S FAM Fluor: | 5'-FAM- GCGATTAGCCGTTAGGATG*A 3' |
| Semi S FAM Fluor: | 5'-FAM- G*CG*AT*TA*GC*CG*TT*AG*GA*TG*A 3' |
| Full S FAM Fluor: | 5'-FAM- G*C*G*A*T*T*A*G*C*C*G*T*T*AG*G*A*T*G*A 3' |
| Unmodified HEX Fluor: | 5'-HEX- GTCGGTGAACAGGTTAGAGA 3' |
| 30 3' S HEX Fluor: | 5'-HEX- GTCGGTGAACAGGTTAGAG*A 3' |
| Semi S HEX Fluor: | 5'-HEX- G*TC*GG*TG*AA*CA*GG*TT*AG*AG*A 3' |
| Full S HEX Fluor: | 5'-HEX- G*T*C*G*G*T*G*A*A*C*A*G*G*T*T*A*G*A*G*A 3' |
| Standard FAM Quencher: | 5' CCTAACGGCTAATCGC -3'Dabsyl |
| 35 Semi S FAM Quencher V1.0: | 5' C*CT*AA*CG*GC*TA*AT*CG*C -3'Dabsyl |
| Semi S FAM Quencher V2.0: | 5' CC*TA*AC*GG*CT*AA*TC*GC -3'Dabsyl |
| Full S FAM Quencher: | 5' C*C*T*A*A*C*G*G*C*T*A*A*T*C*G*C -3'Dabsyl |
| Standard HEX Quencher: | 5' AACCTGTTACCGAC-3'Dabsyl |
| 40 Semi S HEX Quencher V1.0: | 5' AA*CC*TG*TT*CA*CC*GA*C-3'Dabsyl |
| Semi S HEX Quencher V2.0: | 5' A*AC*CT*GT*TC*AC*CG*AC-3'Dabsyl |
| Full S HEX Quencher: | 5' A*A*C*C*T*G*T*T*C*A*C*C*G*A*C-3'Dabsyl |

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range, and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

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The invention will now be described by reference to the following examples which are for illustrative purposes and are not to be construed as a limitation of the scope of the present invention.

EXAMPLE 1

Abbreviations:

FAM: 6-Carboxy Fluorescein
HEX: 2',4',5',7',1,4-hexachlorofluorescein
Dabcyl : non-fluorescent dark quencher

Seven oligonucleotides were designed and their sequences can be found below, where *denotes use of S-modification:

1) Semi S FAM fluorescently-labelled oligonucleotide:
5'-FAM-G*CG*AT*TA*GC*CG*TT*AG*GA*TG*A3'

2) Semi S HEX fluorescently-labelled oligonucleotide: 5'-HEX-
G*TC*GG*TG*AA*CA*GG*TT*AG*AG*A3'

3) Standard FAM Quencher: 5' CCTAACGGCTAATCGC -3'Dabsyl

4) Standard HEX Quencher: 5'AACCTGTTCACCGAC-3'Dabsyl

5) Allele specific primer 1:
5'GCGATTAGCCGTTAGGATGACTGAGTGCAGGTTTCAGACGTCC3'

6) Allele specific primer 2:
5'GTCGGTGAACAGGTTAGAGACTGAGTGCAGGTTTCAGACGTCT3'

7) Common reverse primer: 5' CTCCCTTCCACCTCCGTACCAT3'

It will be noted that in this example the FAM-labelled quenching primer has a 16mer oligonucleotide sequence and is more than 2 nucleotides shorter than the FAM-labelled primer/reporter probe and similarly the HEX-labelled quenching primer has a 15mer oligonucleotide sequence and is more than

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