demonstrated see, for example, Nucl. Acids Res. (2003) 31 (3): e7. doi: 10.1093/nar/gng007. Taq DNA polymerase possesses no 3'→5' Exonuclease (Kenneth R. Tindall, Thomas A. Kunkel, Biochemistry, 1988, 27 (16), p 6008-6013). Enhanced discrimination of single nucleotide polymorphisms by phosphorothioate modification in the presence of a proof-reading polymerase has also been reported. Phosphorothioation increases specificity, reducing incidences of primer-dimer interactions, however it is reported that 3'nuclease functionality is required for the improvement to PCR and it has been demonstrated that in conjunction with Allele Specific PCR the use of S-oligos offer no benefit when used in conjunction with Taq DNA polymerase see, for example, Zhang, J. and Li, K. (2003) Single-Base Discrimination Mediated by Proofreading 3' Phosphorothioate-Modified Primers. Molecular Biotechnology 25, 223-227.

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. Contrary to conventional scientific knowledge the present invention is based on the finding that S-oligos can be used successfully, and result in improvements, in nucleic acid detection assay systems.

SUMMARY ~~OF THE INVENTION~~

According to the ~~invention~~disclosure there is provided a method for reducing non-specific amplification and/or formation of primer-dimers in a template dependent primer extension reaction comprising conducting a primer extension reaction in the presence of a polymerase lacking 3'→5' nuclease activity, wherein one or more of the primers contains at least one phosphorothioate group.

In one aspect, there is provided 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.

In one aspect, there is provided 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:
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 Tm, 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 Tm that is at or below the Ta 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 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 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
c) measuring the detectable signal that is generated when the complementary sequence is hybridised to the first single-labelled oligonucleotide sequence.

Kits and compositions for use in such methods are also provided.

In one aspect, there is provided 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.

In one aspect, there is provided a method or kit as defined herein, for use in allele specific PCR based SNP Genotyping.

In one aspect, there is provided a method or kit as defined herein, 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.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a simple reaction schema for direct detection of a DNA sequence embodying the method of the present invention.

Figure 2 is a simple reaction schema for indirect (real-time) detection of a DNA sequence embodying the method of the present invention.

Figure 3 is a simple reaction schema for indirect (end-point) detection of a DNA sequence in SNP Genotyping embodying the method of the present invention.

Figure 4 shows data generated using the assay described in Example 1 below.

Figure 5 shows a comparison of data generated in assay systems using oligonucleotide sequences containing phosphorothioate groups and non-phosphorothioate containing oligonucleotides as described in Example 2 below.

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 Tm (melting temperature), optionally
a) wherein one of the first and second oligonucleotide sequences has a Tm that is at or below the Ta (annealing temperature) of the primer extension reaction, or

b) wherein one of the first and second oligonucleotide sequences has a Tm that is above the Ta 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:

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 Tm, 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 Tm that is at or below the Ta 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 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 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

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 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 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 Tm that is above the Ta.

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

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. 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.

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.

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.

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.

15. A method or kit according to any one of the preceding claims, wherein the fluorescent quench oligo pairs are:

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.

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.

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

4. The method or kit according to claim 3, wherein one of the first and second oligonucleotide sequences has a Tm that is at or below the Ta (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 Tm that is above the Ta of the primer extension reaction.

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:

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 Tm, 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

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the first and second oligonucleotide sequences being of a Tm that is at or below the Ta 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 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 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

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.

8. A method according to any one of claims 1, 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.

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

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 quenched pair.

11. A method or kit 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.

12. A method or kit according to any one of the preceding claims wherein 20-80% of the bases of phosphorothioate group containing oligonucleotide(s) are phosphorothioates.

13. A method or kit according to any one of the preceding claims, for use in allele specific PCR based SNP Genotyping.

14. A method or kit according to any one of the preceding claims, for monitoring the production of an amplicon via the 5' nuclease assay.

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15. A method according to claim 14, wherein the 5' nuclease assay is being employed to perform allelic discrimination reactions.

16. A method according to any one of claims 1, 3 to 15, wherein the primer extension product is monitored via the use of hybridisation only.

17. A method according to any one of the preceding claims, wherein the primer extension product is monitored via the use of hybridisation only post PCR.

18. A method or kit according to any one of claims 1, to 17, wherein the fluorescent quench oligo pairs range from 6 bp to 100 bp.

19. A method or kit according to claim 18, wherein the fluorescent quench oligo pairs range from 6 bp to 100 bp but are not matched in length.

20. 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.

21. A method or kit according to any one of the preceding claims, wherein the fluorescent quench oligo pairs are labelled one of the pair with a fluorophore and the other with a non fluorescent quenching molecule.

22. A method or kit according to any one of the preceding claims, wherein the fluorescent quench oligo pairs are modified to be resistant to nuclease degradation.

23. A method or kit according to any one of the preceding claims, wherein the fluorescent quench oligo pairs are labelled with molecules that are distance sensitive.

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