

Claims:

1. A method of detecting a target polynucleotide sequence in a given nucleic acid analyte present in a sample, the method comprising the steps of:
 - (a) introducing one or more nucleic acid analytes to a first reaction mixture
5 comprising:
 - i. a single-stranded probe oligonucleotide A_0 ;
 - ii. a pyrophosphorolysing enzyme; and
 - iii. a ligase;wherein the analyte anneals to the single-stranded probe oligonucleotide A_0 to create a first
10 intermediate product which is at least partially double-stranded and in which the 3' end of A_0 forms a double-stranded complex and A_0 is pyrophosphorylated in the 3'-5' direction from the 3' end to create at least a partially digested strand A_1 and A_1 undergoes ligation to form A_2 ;
 - (b) detecting a signal derived from the products of the previous step, wherein the
15 products are A_2 or a portion thereof, or multiple copies of A_2 or multiple copies of a portion thereof, and inferring therefrom the presence or absence of the polynucleotide target sequence in the analyte.
2. A method as claimed in claim 1 further characterised in that the first reaction mixture further
20 comprises a source of pyrophosphate ions.
3. A method as claimed in claim 1 or claim 2 further characterised in that the first reaction mixture further comprises at least one single-stranded primer oligonucleotide that is substantially complementary to a portion of A_0 and deoxyribonucleotide triphosphates (dNTPs).
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4. A method as claimed in claim 3 further characterised in that the first reaction mixture further comprises an amplification enzyme.
5. A method as claimed in claim 1 or claim 2 further characterised in that the products of step
30 (a) are introduced to a second reaction mixture prior to step (b), said second reaction mixture comprising at least one single-stranded primer oligonucleotide and dNTPs.
6. A method as claimed in claim 5 further characterised in that the second reaction mixture

further comprises an amplification enzyme.

7. A method as claimed in claim 1 or claim 2 further characterised in that the first reaction mixture further comprises:

- 5 one or more ligases; and
 two or more LCR probe oligonucleotides that are complementary to adjacent sequences on A_2 , wherein when the probes are successfully annealed to A_2 the 5' phosphate of one LCR probe is directly adjacent to the 3'OH of the other LCR probe;

wherein in the presence of A_2 the two LCR probes will successfully anneal to A_2 and be ligated
10 together to form one oligonucleotide molecule which then acts as a new target for second-round covalent ligation, leading to geometric amplification of the target of interest, in this case A_2 , which is then detected.

8. A method as claimed in claim 1 or claim 2 further characterised in that the products of step
15 (a) are introduced to a second reaction mixture prior to step (b), said second reaction mixture comprising:

- one or more ligases; and
 two or more LCR probe oligonucleotides that are complementary to adjacent sequences on A_2 , wherein when the probes are successfully annealed to A_2 the 5' phosphate of one
20 LCR probe is directly adjacent to the 3'OH of the other LCR probe;

wherein in the presence of A_2 the two LCR probes will successfully anneal to A_2 and be ligated together to form one oligonucleotide molecule which then acts as a new target for second-round covalent ligation, leading to geometric amplification of the target of interest, in this case A_2 which is then detected.

25 9. A method as claimed in claim 1 or claim 2 further characterised in that the first reaction mixture further comprises:

- an oligonucleotide complementary to a region of A_2 including the site of ligation, comprising one or multiple fluorophores arranged such that their fluorescence is quenched either by their proximity to each other or to one or more fluorescence
30 quenchers;

 a double strand specific DNA digestion enzyme;

wherein, in the presence of A_2 , the labelled oligonucleotide is digested such that the fluorophores are separated from each other or from their corresponding quenchers, and a fluorescent signal, and hence the presence of A_2 , is detectable.

- 5 10. A method as claimed in claim 1 or claim 2 further characterised in that the products of step (a) are introduced to a second reaction mixture prior to step (b), said second reaction mixture comprising:

10 an oligonucleotide complementary to a region of A_2 including the site of ligation, comprising one or multiple fluorophores arranged such that their fluorescence is quenched either by their proximity to each other or to one or more fluorescence quenchers;

a double strand specific DNA digestion enzyme;

- 15 wherein, in the presence of A_2 , the labelled oligonucleotide is digested such that the fluorophores are separated from each other or from their corresponding quenchers, and a fluorescent signal, and hence the presence of A_2 , is detectable.

- 20 11. A method as claimed in any one of claims 1 to 6 further characterised in that the partially digested strand A_1 is circularised through ligation of its 3' and 5' ends to create an oligonucleotide A_2 .

- 25 12. A method as claimed in any one of claims 1 to 6 or claim 11 further characterised in that the first reaction mixture further comprises a ligation probe oligonucleotide C and that the partially digested strand A_1 is ligated at the 3' end to the 5' end of C to create an oligonucleotide A_2 .

13. A method as claimed in claim 12 further characterised in that the oligonucleotide C further comprises a 3' or internal modification protecting it from 3'-5' exonuclease digestion

- 30 14. A method as claimed in any one of claims 11 to 13 further characterised in that ligation occurs:
during step (a); or
during step (b); or
inbetween steps (a) and (b).

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15. A method as claimed in any one of claims 1 to 6 or 11 to 14 further characterised in that the first or second reaction mixture further comprises a splint oligonucleotide D.
16. A method as claimed in claim 15 further characterised in that D comprises an oligonucleotide region complementary to the 3' end of A_1 and a region complementary to either the 5' end of an oligonucleotide C or to the 5' end of A_1 .
17. A method as claimed in claim 15 or claim 16 further characterised in that D is unable to undergo extension against A_1 by virtue of either a 3' modification or through a mismatch between the 3' end of D and the corresponding region of A_1 .
18. A method as claimed in any preceding claim further characterised in that the first reaction mixture further comprises a 5'-3' exonuclease and wherein the 5' end of A_0 is rendered resistant to 5'-3' exonuclease digestion.
19. A method as claimed in any preceding claim further characterised in that, prior to step (a) the nucleic acid analytes present in a sample undergo amplification and wherein after amplification of the given nucleic acid analytes and prior to addition of the first reaction mixture, the sample is further treated with a proteinase.
20. A method as claimed in any preceding claim further characterised in that the first reaction mixture further comprises a phosphatase or phosphohydrolase.
21. A method as claimed in any preceding claim further characterised in that prior to or during step (b) the products of the previous step are treated with a pyrophosphatase.
22. A method as claimed in any preceding claim further characterised in that prior to or during step (b) the products of the previous step are treated with an exonuclease.
23. A method as claimed in any one preceding claim further characterised in that the enzyme which performs pyrophosphorolysis of A_0 to form partially digested strand A_1 also amplifies A_2 .

24. A method as claimed in any one of the preceding claims further characterised in that detection is achieved using one or more oligonucleotide fluorescent binding dyes or molecular probes.
25. A method as claimed in claim 24 further characterised in that an increase in signal over time resulting from the generation of amplicons of A_2 is used to infer the concentration of the target sequence in the analyte.
26. A method as claimed in any of the previous claims further characterised in that multiple probes A_0 are employed, each selective for a different target sequence and each including an identification region, and further characterised in that the amplicons of A_2 include this identification region and therefore the target sequences present in the analyte, are inferred through the detection of the identification region(s).
27. A method as claimed in claim 26 further characterised in that detection of the identification regions(s) is carried out using molecular probes or through sequencing.
28. A method as claimed in claim 24 further characterised in that the final step of the method further comprises the steps of:
- labelling the products of step (b) using one or more oligonucleotide fluorescent binding dyes or molecular probes;
 - measuring the fluorescent signal of the products;
 - exposing the products to a set of denaturing conditions; and identifying the polynucleotide target sequence in the analyte by monitoring changes in the fluorescent signal of the products during exposure to the denaturing conditions.
29. A method as claimed in claims 1 to 28 further characterised in that the one or more nucleic acid analytes are split into multiple reaction volumes, each volume having a one or more probe oligonucleotide A_0 , introduced to detect different target sequences.
30. A method as claimed in claims 1 to 29 further characterised in that the different probes A_0

comprise a common priming site, allowing a single primer or single set of primers to be used for amplification.