

In one aspect, the present disclosure relates to a method of detecting a target polynucleotide sequence in a 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 comprising:

- 5 i. a single-stranded probe oligonucleotide A_0 comprising a 3' end region complementary to the target polynucleotide sequence;
- 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 wherein the 3' end of A_0 forms a double-stranded complex with the analyte and wherein A_0 is pyrophosphorylsed in the 3'-5' direction from the 3' end to create at least a partially digested strand A_1 and A_1 undergoes ligation using a splint to form oligonucleotide A_2 , wherein the target polynucleotide sequence functions as the splint and wherein undergoing ligation includes:

15 ligation of the 3' end of A_1 to the 5' end of A_1 to form a circular construct; or ligation of the 3' end of A_1 to the 5' end of a ligation probe oligonucleotide C; and

(b) detecting a signal derived from the products of step (a) and inferring therefrom the presence or absence of the target polynucleotide sequence in the analyte, wherein the products of step (a) comprise A_2 , a portion of A_2 , multiple copies of A_2 , or multiple copies of a portion of A_2 .

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In another aspect, the present disclosure relates to a method of detecting a target polynucleotide sequence in a 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 comprising: i. a single-stranded probe oligonucleotide A_0 comprising a 3' end region complementary to the target
25 polynucleotide sequence; ii. a pyrophosphorolysing enzyme; and iii. a ligase; wherein the analyte anneals to the single-stranded probe oligonucleotide A_0 to create a first intermediate product which is at least partially double-stranded, and wherein the 3' end of A_0 forms a double-stranded complex with the analyte and wherein A_0 is pyrophosphorylsed in the 3'-5' direction from the 3' end to create at least a partially digested strand A_1 , wherein the products of step (a) are introduced to a second reaction mixture, said second reaction mixture comprising: i. at least one
30 single-stranded primer oligonucleotide that is substantially complementary to a portion of A_2 ; ii. dNTPs; and iii. an amplification enzyme; wherein at least one of the first reaction mixture and the

second reaction mixture further comprises oligonucleotide D, wherein oligonucleotide D comprises an oligonucleotide region complementary to the 3' end of A₁ and a region complementary to either the 5' end of oligonucleotide C or to the 5' end of A₁; and A₁ undergoes ligation using oligonucleotide D as a splint to form oligonucleotide A₂ and wherein undergoing ligation includes: ligation of the 3' end of A₁ to the 5' end of A₁ to form a circular construct; or ligation of the 3' end of A₁ to the 5' end of a ligation probe oligonucleotide C; and (b) detecting a signal derived from the products of step (a) and inferring therefrom the presence or absence of the target polynucleotide sequence in the analyte, wherein the products of step (a) comprise A₂, a portion of A₂, multiple copies of A₂, or multiple copies of a portion of A₂.

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Brief Description of Drawings

Fig. 1: Protocols for simplified polynucleotide sequence detection methods.

Fig. 2: A graph comparing the level of fluorescence detected (representing the presence of a particular target analyte sequence) when the 5'-3' exonuclease digestion step happens during the pre-amplification step and when it is moved to the pyrophosphorolysis/ligation step of the protocol (as in protocols 3-5). In this example, the 5'-3' exonuclease is Lambda.

Fig. 3: The inventors have tested the method of Protocol 3 of the current invention using a range of different PPL enzymes. Fig. 3 (A) shows detection of 1% MAF T790M using Mako, Klenow and Bsu. Fig. 3(B) shows the detection of 0.5% MAF T790M using Bst LF at a range of different Ppi concentrations. All four enzymes performed very well even without extended optimisation.

Fig. 4: Results for the detection of 1% MAF, T790M, using the methods of Protocol 4 of the current invention using four different pyrophosphorolysing (PPL) enzymes: Mako, Klenow, Bsu and Bst LF.

Fig. 5: Graphs showing the level of fluorescence detected (representing the presence of particular target analyte sequence) in the presence of 0.5%, 0.10% and 0.05% MAF Exon19 del_6223 using the methods according to Protocol 1 and Protocol 4.

Fig. 6: The inventors have detected EGFR exon 20 T790M at 0.10%, 0.50% and 1% MAF according to Protocol 4.

Fig. 7: Detection of the EGFR exon 20 mutation T790M at 1% MAF with and without the use of an exonuclease in the RCA step.

- 5 Fig. 8: The inventors have investigated what effect the PPL:RCA mix ratio has on the intensity of signal detected for 0.5% MAF EGFR exon 20 T790M, the results of which are shown in Fig. 8. As can be seen a ratio of 1:2 PPL:RCA mix results in the lowest signal intensity but at the earliest time point. This is followed closely in time by 1:4 PPL:RCA mix which has a greater signal intensity. The largest signal intensity is seen for 1:8 PPL:RCA mix at the latest time point in the reaction.

Claims:

1. A method of detecting a target polynucleotide sequence in a 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
 - i. a single-stranded probe oligonucleotide A_0 comprising a 3' end region complementary to the target polynucleotide sequence;
 - ii. a pyrophosphorolysing enzyme; and
 - iii. a ligase;
 - wherein the analyte anneals to the single-stranded probe oligonucleotide A_0 to create a first intermediate product which is at least partially double-stranded, and wherein the 3' end of A_0 forms a double-stranded complex with the analyte and wherein A_0 is pyrophosphorylsed in the 3'-5' direction from the 3' end to create at least a partially digested strand A_1 and A_1 undergoes ligation using a splint to form oligonucleotide A_2 , wherein the target polynucleotide sequence functions as the splint and wherein undergoing ligation includes:
 - ligation of the 3' end of A_1 to the 5' end of A_1 to form a circular construct; or ligation of the 3' end of A_1 to the 5' end of a ligation probe oligonucleotide C; and
 - (b) detecting a signal derived from the products of step (a) and inferring therefrom the presence or absence of the target polynucleotide sequence in the analyte, wherein the products of step (a) comprise A_2 , a portion of A_2 , multiple copies of A_2 , or multiple copies of a portion of A_2 .
2. A method of detecting a target polynucleotide sequence in a 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
 - i. a single-stranded probe oligonucleotide A_0 comprising a 3' end region complementary to the target polynucleotide sequence;
 - ii. a pyrophosphorolysing enzyme; and
 - iii. a ligase;
 - wherein the analyte anneals to the single-stranded probe oligonucleotide A_0 to create a first intermediate product which is at least partially double-stranded, and wherein the 3' end of A_0 forms a double-stranded complex with the analyte and wherein A_0 is pyrophosphorylsed in the 3'-5' direction from the 3' end to create at least a partially digested strand A_1 , wherein the products

of step (a) are introduced to a second reaction mixture, said second reaction mixture comprising:

- i. at least one single-stranded primer oligonucleotide that is substantially complementary to a portion of A_2 ;
- ii. dNTPs; and
- 5 iii. an amplification enzyme;

wherein at least one of the first reaction mixture and the second reaction mixture further comprises oligonucleotide D, wherein oligonucleotide D comprises an oligonucleotide region complementary to the 3' end of A_1 and a region complementary to either the 5' end of oligonucleotide C or to the 5' end of A_1 ; and A_1 undergoes ligation using oligonucleotide D as a
10 splint to form oligonucleotide A_2

and wherein undergoing ligation includes:

- ligation of the 3' end of A_1 to the 5' end of A_1 to form a circular construct; or
- ligation of the 3' end of A_1 to the 5' end of a ligation probe oligonucleotide C;
- 15 and
- (b) detecting a signal derived from the products of step (a) and inferring therefrom the presence or absence of the target polynucleotide sequence in the analyte, wherein the products of step (a) comprise A_2 , a portion of A_2 , multiple copies of A_2 , or multiple copies of a portion of A_2 .

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3. The method of claim 1 or claim 2, wherein the first reaction mixture further comprises a source of pyrophosphate ions.
4. The method of claim 1 or claim 2 wherein the first reaction mixture further comprises at least
25 one single-stranded primer oligonucleotide that is substantially complementary to a portion of A_0 and deoxyribonucleotide triphosphates (dNTPs).
5. The method of claim 4, wherein the first reaction mixture further comprises an amplification enzyme.
- 30 6. The method of claim 4, wherein the dNTPs are hot start dNTPs.

7. The method of claim 2, wherein D is unable to undergo extension against A_1 by virtue of a 3' modification of D, or through a mismatch between the 3' end of D and the corresponding region of A_1 .
- 5 8. The method of claim 1 or claim 2, wherein the partially digested strand A_1 is circularised through ligation of its 3' and 5' ends to create an oligonucleotide A_2 .
9. The method of claim 8, wherein ligation occurs:
during step (a);
10 during step (b); or
between steps (a) and (b).
10. The method of claim 1 or claim 2, wherein the first reaction mixture further comprises a 5'-3' exonuclease, and wherein the 5' end of A_0 is resistant to 5'-3' exonuclease digestion.
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11. The method of claim 2, wherein the first reaction mixture further comprises the ligation probe oligonucleotide C, and the partially digested strand A_1 is ligated at the 3' end to the 5' end of C to create an oligonucleotide A_2 .
- 20 12. The method of claim 11, wherein ligation occurs:
during step (a);
during step (b); or
between steps (a) and (b).
- 25 13. The method of claim 12, wherein the first reaction mixture further comprises a 5'-3' exonuclease, and wherein the 5' end of A_0 is resistant to 5'-3' exonuclease digestion.
14. The method of claim 12, wherein the oligonucleotide C further comprises a 3' or internal modification protecting it from 3'-5' exonuclease digestion.
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15. The method of claim 1 or claim 2, wherein the first reaction mixture further comprises a phosphatase or phosphohydrolase.

16. The method of claim 12, wherein prior to or during step (b), the products of step (a) are treated with a pyrophosphatase or an exonuclease.
17. The method of claim 2, wherein the first reaction mixture further comprises the oligonucleotide D.
18. The method of claim 2, wherein D is unable to undergo extension against A_1 by virtue of a 3' modification of D, or through a mismatch between the 3' end of D and the corresponding region of A_1 .
19. The method of claim 1 or claim 2, wherein the enzyme which performs pyrophosphorolysis of A_0 to form partially digested strand A_1 also amplifies A_2 .
20. The method of claim 1 or claim 2, wherein detection is achieved using one or more oligonucleotide fluorescent binding dyes or molecular probes.
21. The method of claim 20, wherein an increase in signal over time resulting from the generation of amplicons of A_2 is used to infer a concentration of the target polynucleotide sequence in the analyte.
22. The method of claim 20, wherein step (b) comprises :
- i. labelling the products of step (a) using one or more oligonucleotide fluorescent binding dyes or molecular probes;
 - ii. measuring the fluorescent signal of the labelled products;
 - iii. exposing the labelled products to a set of denaturing conditions; and
- identifying the target polynucleotide sequence in the analyte by monitoring changes in the fluorescent signal of the labelled products during exposure to the denaturing conditions.
23. The method of claim 1 or claim 2, wherein the first reaction mixture comprises multiple probes A_0 , each selective for a different target polynucleotide sequence of the analyte and each including an identification region, wherein amplicons of A_2 include at least one of the identification regions, and wherein the presence of the target polynucleotide sequences present in the analyte are inferred through the detection of the identification region(s).

24. The method of claim 23, wherein detection of the identification regions(s) is carried out using molecular probes or through sequencing.
- 5 25. The method of claim 23, wherein the multiple probes A_0 comprise a common priming site, allowing a single primer or single set of primers to be used for amplification.
- 10 26. The method of claim 1 or claim 2, wherein the one or more nucleic acid analytes are split into multiple reaction volumes, each volume being introduced into a respective first reaction mixture having one or more probe oligonucleotides A_0 selective for different target sequences.