

design and further limits the efficacy of multiplexing. This is particularly relevant when searching for panels of SNPs as is a common requirement in cancer testing/screening or companion diagnostics.

5 US2006/110765 A1 (Wang et al) teaches enzymatic cleavage at a mismatch, which is typically an inefficient and not a highly specific reaction. Moreover, off target hybridisation of the probes as disclosed in Wang et al to similar sequences in a sample would result in false positive results because of the use of cleavage at mismatches. It would also not be possible to distinguish
10 all result in cleavage and amplification of the same probe. The teachings of Wang et al would therefore result in low sensitivity and specificity of the reaction scheme. In contrast, the technical effect of the method as disclosed by the present invention provides a fast, efficient method with high specificity to dsDNA, which can be effectively blocked by a mismatch. In addition, the method of the present invention is extremely specific to the targeted genetic variant, enabling
15 discrimination between different variants in the same or near-neighbouring positions.

US2009/239283 A1 (Liu et al) teaches the use of non-extendable 3' ends which are removed by pyrophosphorolysis, necessitating the genetic engineering of custom polymerases capable of removing the 3' blocking modification. In contrast the present invention utilises the natural
20 pyrophosphorolysis activity inherent in existing polymerases and does not use 3' blocking modifications. The method as disclosed in Liu et al also relies on the removal of only the terminal base from a fraction of the probe to enable subsequent amplification, and is limited to this embodiment by the use of 3' blocking modification. In contrast, the methods disclosed in the present invention enables embodiments, in which progressive removal of multiple bases from the
25 probe is required to set off the reaction, making it substantially more robust to transient off-target annealing either to the background DNA or to other probes, which can result in the unwanted removal of the terminal base.

Summary of Invention

30 We have now developed a new simplified method which builds on our experience using the pyrophosphorolysis method employed in our earlier patent (PCT/GB2019/052017) to overcome many of these limitations. In doing so, it harnesses the double-strand specificity of pyrophosphorolysis; a reaction which will not proceed efficiently with single-stranded

oligonucleotide substrates or double-stranded substrates which include blocking groups or nucleotide mismatches. The new method is faster, less complex and less expensive to run than that disclosed in PCT/GB2019/052017. Thus, according to the present ~~invention~~disclosure, there is provided a method of detecting a target polynucleotide sequence in a given nucleic acid analyte, 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 ;
- ii. a pyrophosphorolysing enzyme; and
- iii. a ligase

wherein A_0 is pyrophosphorolysed 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 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.

The analytes to which the method of the ~~disclosure~~invention can be applied are those nucleic acids, such as naturally-occurring or synthetic DNA or RNA molecules, which include the target polynucleotide sequence(s) being sought. In one ~~embodiment~~example, the analyte will typically be present in an aqueous solution containing it and other biological material and in one ~~embodiment~~example the analyte will be present along with other background nucleic acid molecules which are not of interest for the purposes of the test. In some ~~embodiment~~examples, the analyte will be present in low amounts relative to these other nucleic acid components. Preferably, for example where the analyte is derived from a biological specimen containing cellular material, prior to performing step (a) of the method some or all of these other nucleic acids and extraneous biological material will have been removed using sample-preparation techniques such as filtration, centrifuging, chromatography or electrophoresis. Suitably, the analyte is derived from a biological sample taken from a mammalian subject (especially a human patient) such as blood, plasma, sputum, urine, skin or a biopsy. In one ~~embodiment~~example, the biological sample will be subjected to lysis in order that the analyte is released by disrupting any cells present. In other ~~embodiment~~examples, the analyte may already be present in free form within the sample itself; for example cell-free DNA circulating in blood or plasma.

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:

- 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 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 using a splint to form oligonucleotide A_2 , wherein the target polynucleotide sequence functions as the splint or the splint includes an oligonucleotide D, 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

(b) ligation of the 3' end of A_1 to the 5' end of a ligation probe oligonucleotide C; and 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 .

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.

5 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.

10 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.

15 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.

20 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 ~~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 comprising:

- 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 in which~~ the 3' end of A_0 forms a double-stranded complex with the analyte and wherein 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 using a splint to form oligonucleotide A_2 , wherein the target polynucleotide sequence functions as the splint or the splint includes an oligonucleotide D, 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) the previous step,~~ wherein the 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 target polynucleotide ~~target~~ 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. ~~The method of A method as claim in~~ claim 1, ~~further characterised in that~~ wherein the first reaction mixture further comprises a source of pyrophosphate ions.

3. ~~The method of A method as claimed in~~ claim 1 ~~or claim 2 further characterised in that~~ wherein 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).

4. The method of ~~A method as claimed in~~ claim 3, ~~further characterised in that~~ wherein the first reaction mixture further comprises an amplification enzyme.

4.5. The method of claim 3, wherein the dNTPs are hot start dNTPs.

5.6. A ~~The method of method as claimed in~~ claim 1 ~~or claim 2~~, wherein 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 at least one single-stranded primer oligonucleotide and dNTPs.

6.7. The method of claim 6, ~~A method as claimed in claim 5~~ further characterised in that wherein the second reaction mixture further comprises an amplification enzyme.

8. The method of claim 6, wherein the first or second reaction mixture further comprises the oligonucleotide D.

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

~~one or more ligases; and~~

~~two or more LCR probe oligonucleotides that are complementary to adjacent sequences on A₂, wherein when the probes are successfully annealed to A₂ the 5' phosphate of one LCR probe is directly adjacent to the 3'OH of the other LCR probe;~~

~~wherein in the presence of A₂ the two LCR probes will successfully anneal to A₂ 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 A₂, which is then detected.~~

~~8. 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:~~

~~one or more ligases; and~~

~~two or more LCR probe oligonucleotides that are complementary to adjacent sequences on A₂, wherein when the probes are successfully annealed to A₂ 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 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.

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 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.

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:

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;

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.

~~11.1. A method as claimed in any one of claims 1 to 6 further characterised in that the partially digested strand A_2 is circularised through ligation of its 3' and 5' ends to create an oligonucleotide A_2 .~~

~~12.1. 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_2 is ligated at the 3' end to the 5' end of C to create an~~

~~oligonucleotide A₂.~~

~~13.1. 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~~

5 ~~14.1. 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).~~

10 ~~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.~~

15 ~~16.9. The method of claim 8, wherein A method as claimed in claim 15 further characterised in that D comprises an oligonucleotide region complementary to the 3' end of A₁ and a region complementary to either the 5' end of an oligonucleotide C or to the 5' end of A₁.~~

20 ~~17.10. The method of claim 8, wherein A method as claimed in claim 15 or claim 16 further characterised in that D is unable to undergo extension against A₁ by virtue of either a 3' modification of D, or through a mismatch between the 3' end of D and the corresponding region of A₁.~~

25 ~~11. The method of claim 1, wherein A method as claimed in any one of claims 1 to 6 further characterised in that the partially digested strand A₁ is circularised through ligation of its 3' and 5' ends to create an oligonucleotide A₂.~~

30 ~~12. The method of claim 11, wherein 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~~

in between steps (a) and (b).

~~18-13. The method of claim 1, wherein the 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 A₀ is ~~rendered~~-resistant to 5'-3' exonuclease digestion.

~~14. The method of claim 1, wherein 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~~the ligation probe oligonucleotide C, and ~~that the~~ partially digested strand A₁ is ligated at the 3' end to the 5' end of C to create an oligonucleotide A₂.

~~15. The method of claim 14, wherein ligation occurs:~~

~~during step (a);~~

~~during step (b); or~~

~~between steps (a) and (b).~~

~~16. The method of claim 15, wherein the first reaction mixture further comprises a 5'-3' exonuclease, and wherein the 5' end of A₀ is resistant to 5'-3' exonuclease digestion.~~

~~17. The method of claim 15, wherein 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.

~~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.~~

~~20-18. The method of claim 1, wherein 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.~~

~~22-19. The method of claim 15, wherein A method as claimed in any preceding claim further characterised in that~~ prior to or during step (b), the products of ~~the previous step~~ step (a) are treated with a pyrophosphatase or an exonuclease.

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20. The method of claim 1, wherein the first reaction mixture further comprises the oligonucleotide D.

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21. The method of claim 20, wherein the splint includes the oligonucleotide D, and wherein 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.

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22. The method of claim 21, wherein D is unable to undergo extension against A₁ by virtue of a 3' modification of D, or through a mismatch between the 3' end of D and the corresponding region of A₁.

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~~23. The method of claim 1, wherein A method as claimed in any one preceding claim further characterised in that~~ the enzyme which performs pyrophosphorolysis of A₀ to form partially digested strand A₁ also amplifies A₂.

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~~24. The method of claim 1, wherein 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.

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~~25. The method of claim 24, wherein A method as claimed in claim 24 further characterised in that~~ an increase in signal over time resulting from the generation of amplicons of A₂ is used to infer ~~the a~~ concentration of the target polynucleotide sequence in the analyte.

~~26.1. A method as claimed in any of the previous claims further characterised in that multiple probes A₀ are employed, each selective for a different target sequence and each including an identification region, and further characterised in that the amplicons of A₂ include this identification region and therefore the target sequences present in the analyte, are inferred through the detection of the identification region(s).~~

~~27.1. 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.~~

5 ~~28.26. The method of claim 24, wherein step (b) comprises A method as claimed in claim 24 further characterised in that the final step of the method further comprises the steps of:~~

- i. labelling the products of step (ba) using one or more oligonucleotide fluorescent binding dyes or molecular probes;
 - ii. measuring the fluorescent signal of the labelled products;
 - 10 iii. exposing the labelled products to a set of denaturing conditions; and
- identifying the target polynucleotide ~~target~~-sequence in the analyte by monitoring changes in the fluorescent signal of the labelled products during exposure to the denaturing conditions.

15 ~~27. The method of claim 1, wherein the first reaction mixture comprises A method as claimed in any of the previous claims further characterised in that multiple probes A₀ are employed, each selective for a different target polynucleotide sequence of the analyte and each including an identification region, and further characterised in that the ~~wherein~~ amplicons of A₂ include ~~this~~ at least one of the identification regions, and therefore ~~wherein the~~ presence of the target polynucleotide sequences present in the analyte, are inferred through the~~

20 detection of the identification region(s).

~~28. The method of claim 27, wherein 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.~~

25 ~~29.1. 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 A₀, introduced to detect different target sequences.~~

30 ~~30.29. The method of claim 27, wherein the A method as claimed in claims 1 to 29 further characterised in that the different multiple probes A₀ comprise a common priming site, allowing a single primer or single set of primers to be used for amplification.~~

- 5 30. The method of claim 1, wherein the ~~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 A₀~~ being introduced into a respective first reaction mixture having one or more probe oligonucleotides A₀ selective for introduced to detect different target sequences.