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.

Summary

In one aspect, the present disclosure refers to a method of detecting a target polynucleotide sequence in a nucleic acid analyte, the method comprising:

a. annealing the analyte to a single-stranded probe oligonucleotide $A_0$ to create a first intermediate product which is at least partially double-stranded and in which the 3' end of $A_0$ forms a double-stranded complex with the target polynucleotide sequence;
b. pyrophosphorolysing the first intermediate product with a pyrophosphorolysing enzyme in the 3'-5' direction from the 3' end of $A_0$ to create partially digested strand $A_1$ and the analyte;
c. (i) annealing $A_1$ to a single-stranded trigger oligonucleotide B and extending the $A_1$ strand in the 5'-3' direction against B; or (ii) circularising $A_1$ through ligation of its 3' and 5' ends; or (iii) ligating the 3' end of $A_1$ to the 5' end of a ligation probe oligonucleotide C; in each case to create an oligonucleotide $A_2$;
d. priming $A_2$ with at least one single-stranded primer oligonucleotide and creating multiple copies of $A_2$, or multiple copies of a region of $A_2$; and
e. detecting a signal derived from the multiple copies and inferring therefrom the presence or absence of the target polynucleotide sequence in the analyte.

We have now developed a new method which builds on our experience using the pyrophosphorolysis method employed in our earlier sequencing patents (see for example WO 2016012789) 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. Thus, according to the present disclosure, there is provided a method of detecting a target polynucleotide sequence in a given nucleic acid analyte characterised by the steps of:

a. annealing the analyte to a single-stranded probe oligonucleotide $A_0$ to create a first intermediate product which is at least partially double-stranded and in which the 3' end of $A_0$ forms a double-stranded complex with the analyte target sequence;

b. pyrophosphorolysing the first intermediate product with a pyrophosphorolysing enzyme in the 3'-5' direction from the 3' end of $A_0$ to create partially digested strand $A_1$ and the analyte;

c. (i) annealing $A_1$ to a single-stranded trigger oligonucleotide B and extending the $A_1$ strand in the 5'-3' direction against B; or (ii) circularising $A_1$ through ligation of its 3' and 5' ends; or (iii) ligating the 3' end of $A_1$ to the 5' end of a ligation probe oligonucleotide C; in each case to create an oligonucleotide $A_2$;

d. priming $A_2$ with at least one single-stranded primer oligonucleotide and creating multiple copies of $A_2$, or a region of $A_2$; and

e. detecting a signal derived from the multiple copies and inferring therefrom the presence or absence of the polynucleotide target sequence in the analyte.

The analytes to which the method of the present disclosure 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 example, the analyte will typically be present in an aqueous solution containing it and other biological material and in one 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 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 example, the biological sample will be subjected to lysis in order that the analyte is released by disrupting any cells present. In other examples, the analyte may already be present in free form within the sample itself; for example cell-free DNA circulating in blood or plasma.

Brief Description of Drawings

Fig. 1: Gel electrophoresis image of the reaction products of Example 1. It can be seen that in the presence of oligonucleotide 2, oligonucleotide 1 is degraded to the length at which it melts from oligonucleotide 2, leaving a shortened oligonucleotide approximately 50 nucleotides in length. Conversely, in the presence of oligonucleotide 3, no pyrophosphorolysis is observed due to the single nucleotide mismatch at the 3' end of oligonucleotide 1. In the presence of oligonucleotides 4-6, pyrophosphorolysis of oligonucleotide 1 proceeds to the position of the single base mismatch at which point it stops, leaving a shortened oligonucleotide which is not further degraded.

Fig. 2: Gel electrophoresis image of the reaction products of Example 2. It can be seen that the shortened oligonucleotide (oligonucleotide 1) is efficiently circularised by the ligation reaction and survives subsequent exonuclease digestion, while the un-shortened oligonucleotide (oligonucleotide 2) is not circularised and is efficiently digested.

Fig. 3: Gel electrophoresis image of the reactions products of Example 3. It can be seen that when the shortened oligonucleotide was present, and circularised, in Example 2 a large amount of product is produced by this amplification. Conversely, when the un-shortened oligonucleotide was present in Example 2, and no circularisation took place, there was no observable amplification of DNA.

Claims:

1. A method of detecting a target polynucleotide sequence in a nucleic acid analyte, the method comprising:
    a. annealing the analyte to a single-stranded probe oligonucleotide $A_0$ to create a first intermediate product which is at least partially double-stranded and in which the 3' end of $A_0$ forms a double-stranded complex with the target polynucleotide sequence;
    b. pyrophosphorolysing the first intermediate product with a pyrophosphorolysing enzyme in the 3'-5' direction from the 3' end of $A_0$ to create partially digested strand $A_1$ and the analyte;
    c. (i) annealing $A_1$ to a single-stranded trigger oligonucleotide B and extending the $A_1$ strand in the 5'-3' direction against B; or (ii) circularising $A_1$ through ligation of its 3' and 5' ends; or (iii) ligating the 3' end of $A_1$ to the 5' end of a ligation probe oligonucleotide C; in each case to create an oligonucleotide $A_2$;
    d. priming $A_2$ with at least one single-stranded primer oligonucleotide and creating multiple copies of $A_2$, or multiple copies of a region of $A_2$; and
    e. detecting a signal derived from the multiple copies and inferring therefrom the presence or absence of the target polynucleotide sequence in the analyte.
2. The method of claim 1, wherein c(ii) or c(iii) is employed , and $A_1$ is first extended in the 5'-3' direction prior to ligation.
3. The method of claim 1, wherein the ligation is performed through addition of a further splint oligonucleotide D to which $A_1$ anneals prior to ligation, wherein 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 $A_1$.
4. The method of claim 3, wherein the oligonucleotide 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 $A_1$.
5. The method of claim 1, wherein a reaction mixture produced from step (c) is treated with an exonuclease after step (c) to substantially digest any non-ligated nucleic acid material, and wherein if c(iii) is employed, the oligonucleotide C further comprises a 3' or internal modification protecting it from 3'-5' exonuclease digestion.

6. The method of claim 5, wherein the exonuclease is deactivated prior to step (d).
7. The method of claim 1, wherein c(i) is employed, and B comprises (i) an oligonucleotide region complementary to the 3' end of $A_1$ and (ii) a flanking oligonucleotide region at its 5' end which is not substantially complementary to $A_0$ or the target polynucleotide sequence, and wherein one of the primer oligonucleotides used in step (d) anneals to the extended region of $A_2$.
8. The method of claim 1, wherein the probe oligonucleotide $A_0$ has a 5' end which is resistant to exonucleolysis, and wherein after steps (a) and (b), a reaction medium produced therefrom is treated with a 5'-3' exonuclease to substantially remove any nucleic acid molecules which are not rendered resistant to this exonucleolysis.
9. The method of claim 1, wherein step (b) is carried out in the presence of a phosphatase.
10. The method of claim 1, wherein steps (a) and (b) are iterated to create multiple copies of partially digested probe $A_1$ from the analyte.
11. The method of claim 1, wherein the pyrophosphorolysis reaction is stopped after step (b) through addition of a pyrophosphatase.
12. The method of claim 1, wherein step (e) comprises detecting a signal derived from the multiple copies using one or more oligonucleotide binding dyes or molecular probes.
13. The method of claim 1, wherein steps (d) and (e) are performed concurrently.
14. The method of claim 13, wherein an increase in signal over time resulting from generation of amplicons in step (d) is used to infer a concentration of the target polynucleotide sequence in the analyte.
15. The method of claim 1, wherein the analyte is single-stranded and prior to step (a), the analyte is derived from a biological sample comprising the analyte by: (i) producing amplicons of the analyte by subjecting the biological sample to cycles of amplification; and (ii) digesting the product of step (i) with an exonuclease having 5'-3' exonucleolytic activity, wherein one of the primers employed in step (i) includes an exonuclease blocking group.
16. The method of claim 15, wherein step (i) is carried out using deoxyuridine triphosphate instead of deoxythymidine triphosphate and in the presence of UTP-DNA glycolase.

17. The method of claim 1, wherein the analyte is single stranded and prior to step (a), the analyte is derived from a biological sample comprising the analyte by (i) producing amplicons of the analyte by subjecting the biological sample to cycles of amplification, wherein one of the primers employed is introduced in excess to the other(s).
18. The method of claim 17, wherein the amplification employs a polymerase exhibiting 3'-5' exonuclease activity, wherein after step (i), the product of step (i) is reacted with a proteinase to destroy the polymerase, and wherein the proteinase is then destroyed by heating the product of this reaction.
19. The method of claim 17, wherein the amplification employs a polymerase exhibiting 3'-5' exonuclease activity, wherein after step (i), the product of step (i) is reacted with a proteinase to destroy the polymerase, and wherein the proteinase is then destroyed by heating the product of this reaction.
20. The method of claim 17, wherein step (i) is carried out using deoxyuridine triphosphate instead of deoxythymidine triphosphate and in the presence of UTP-DNA glycolase.
21. The method of claim 1, wherein multiple probes $A_0$ are employed, each selective for a different target polynucleotide sequence and each including an identification region, and wherein the region amplified in step (d) includes this identification region.
22. The method of claim 21, wherein the presence of the target sequences present in the analyte is inferred from the amplicons generated in step (d) through the detection of the identification region(s).
23. The method of claim 22, wherein detection of the identification regions(s) is carried out using molecular probes or through sequencing.
24. The method of claim 23, wherein step (e) further comprises the steps of:
    i. labelling the multiple copies of $A_2$, or the multiple copies of the region of $A_2$ using one or more oligonucleotide fluorescent binding dyes or molecular probes;
    ii. measuring the fluorescent signal of the multiple copies;
    iii. exposing the multiple copies to a set of denaturing conditions; and
    iv. identifying the target polynucleotide sequence in the analyte by monitoring changes in the fluorescent signal of the multiple copies during exposure to the denaturing conditions.

25. The method of claim 21, wherein the different probes $A_0$ comprise a common priming site, allowing a single or single set of primers to be used for amplification in step (d).
26. The method of claim 1, wherein prior to step (a), the analyte is split into multiple reaction volumes, each volume having a different probe oligonucleotide $A_0$ introduced to detect a different target polynucleotide sequence.