for panels of SNPs as is a common requirement in cancer testing/screening or companion diagnostics.

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 between two different genetic variants in the same or near-neighbouring positions as they would 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 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 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 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~~

We have now developed kits and devices for use with a new method which builds on our experience using the pyrophosphorolysis method employed in our earlier patent (see 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. Thus, according to the present ~~invention~~disclosure, there is provided a kit comprising:

(a) a single-stranded probe oligonucleotide A_0, capable of forming a first intermediate product with a target polynucleotide sequence, said intermediate product being at least partially double-stranded;

(b) a ligase;

(c) a pyrophosphorolysing enzyme capable of digesting the first intermediate product in the 3'-5' direction from the end of A_0 to create a partially digested strand A_1;

(d) at least one single-stranded primer oligonucleotide that is substantially complementary to a portion of A_0;

(e) an amplification enzyme;

(f) suitable buffers.

And a device comprising:

at least a fluid pathway between a first region, a second region and a third region,

wherein the first region comprises one or more wells, wherein each well comprises:

dNTPs;

at least one single-stranded primer oligonucleotide;

an amplification enzyme for the initial amplification of DNA present in a sample;

and

wherein the second region comprises one or more wells, wherein each well comprises:

a single-stranded probe oligonucleotide A_0, capable of forming a first intermediate product with a target polynucleotide sequence, said intermediate product being at least partially double-stranded;

a pyrophosphorolysing enzyme capable of digesting the first intermediate product in the 3'-5' direction from the end of A_0 to create a partially digested strand A_1;

at least one source of pyrophosphate ion;

a ligase;

and

wherein the third region comprises one or more wells, wherein each well comprises:

dNTPs;

buffers;

an amplification enzyme;

a means for detecting a signal derived from A_1 or a portion thereof, or multiple copies of A_1 or multiple copies of a portion thereof; and

wherein the wells of the second region or the wells of the third region further comprise at least one single-stranded primer oligonucleotide that is substantially complementary to a portion of A_0.

In one aspect, according to the present disclosure, there is provided a kit comprising:

(a) a single-stranded probe oligonucleotide A_0, capable of forming a first intermediate product with a target polynucleotide sequence, said intermediate product being at least partially double-stranded;

(b) a ligase;

(c) a pyrophosphorolysing enzyme capable of digesting the first intermediate product in the 3'-5' direction from the end of A_0 to create a partially digested strand A_1;

(d) a source of ions to drive the pyrophosphorolysis reaction, wherein optionally the ions are pyrophosphate ions; and

(e) suitable buffers.

The analytes to which the method of the ~~invention~~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 ~~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 (b) 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.

Claims:

1. A kit comprising:

   (a) a single-stranded probe oligonucleotide A_0, capable of forming a first intermediate product with a target polynucleotide sequence, said intermediate product being at least partially double-stranded;

   (b) a ligase;

   (c) a pyrophosphorolysing enzyme capable of digesting the first intermediate product in the 3'-5' direction from the end of A_0 to create a partially digested strand A_1;

   (d) a source of ions to drive the pyrophosphorolysis reaction, wherein optionally the ions are pyrophosphate ions; and

   (e) suitable buffers.

2. A kit as claimed in claim 1, further comprising positive and negative controls.

3. A kit as claimed in any one preceding claim, wherein the 5' end of A_0 is rendered resistant to 5'-3' exonuclease digestion and wherein the kit further comprises a 5'-3' exonuclease.

4. A kit as claimed in any one of claims 1 to 3, wherein the kit further comprises deoxynucleotide triphosphates (dNTPs), a polymerase and buffers for the initial amplification of a target polynucleotide sequence present in a sample.

5. A kit as claimed in claim 4, wherein the kit further comprises a dUTP incorporating high fidelity polymerase, dUTPs and uracil-DNA N-glycosylase (UDG).

6. A kit as claimed in any one of claims 1 to 5, wherein the kit further comprises a proteinase.

7. A kit as claimed in any one of claims 1 to 6, further comprising a ligation probe oligonucleotide C.

8. A kit as claimed in any one of claims 1 to 6, further comprising a splint oligonucleotide D.

9. A kit as claimed any one of claims 1 to 6, further comprising a ligation probe oligonucleotide C and a splint oligonucleotide D.

10. A kit as claimed in claim 7 or 9, wherein the ligation probe oligonucleotide C comprises a 3' or internal modification protecting it from 3'-5' exonuclease digestion and the kit further comprises a 3'-5' exonuclease.

11. A kit as claimed in claim 8, 9 or 10, 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.

12. A kit as claimed in claim 11, wherein 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 or C.

13. A kit as claimed in any one preceding claim, wherein the kit further comprises at least one single-stranded primer oligonucleotide that is substantially complementary to a portion of A_0, an amplification enzyme, dNTPs and one or more oligonucleotide binding dyes or molecular probes.

14. A kit as claimed in any one preceding claim, wherein the kit further comprises multiple A_0, each selective for a different target sequence and each including an identification region.

15. A kit as claimed in any one of claims 1 to 12, wherein the kit further comprises:
    - two or more Ligation Chain Reaction (LCR) probe oligonucleotides that are complementary to adjacent sequences on A_1 wherein when the probes are successfully annealed the 5' phosphate of one LCR probe is directly adjacent to the 3'OH of the other LCR probe; and
    - one or more ligases;

16. A kit as claimed in claim 13, wherein the amplification enzyme is the same as the pyrophosphorolysis enzyme.

17. A kit as claimed in any one of claims 1 to 12, wherein the kit further comprises
    - A ligation probe oligonucleotide C;
    - A splint oligonucleotide D;

wherein C has a 5' phosphate, the 3' end of a splint oligonucleotide D is complementary to the 5' end of C and the 5' end of D is complementary to the 3' end of A_1 such that A_1 and C are capable of being ligated together to form A_2.

18. A kit as claimed in claim 17, wherein the kit further comprises:
    - A hairpin oligonucleotide 1 (HO1) comprising a fluorophore-quencher pair, wherein HO1 is complementary to A_2 and when annealed to A_2 the hairpin structure of HO1 opens and the fluorophore-quencher pair separate; and

- A hairpin oligonucleotide 2 (HO2) comprising a fluorophore-quencher pair, wherein HO2 is complementary to the open HO1 and when annealed to HO1 the hairpin structure of HO2 opens and the fluorophore-quencher pair separate.

19. A kit as claimed in claim 18, wherein the kit further comprises a plurality of HO1 and HO2.

20. A kit as claimed in any one of claims 1 to 12, wherein the kit further comprises:
    - an oligonucleotide A comprising a substrate arm, a partial catalytic core and a sensor arm;
    - an oligonucleotide B comprising a substrate arm, a partial catalytic core and a sensor arm; and
    - a substrate comprising a fluorophore quencher pair;

wherein the sensor arms of oligonucleotides A and B are complementary to flanking regions of A_2 such that in the presence of A_2,oligonucleotides A and B are combined to form a catalytically, multicomponent nucleic acid enzyme (MNAzyme).

21. A kit as claimed in any one of claims 1 to 12, wherein the kit further comprises a partially double-stranded nucleic acid construct wherein:
    - one strand comprises at least one RNA base, at least one fluorophore and wherein a region of this strand is complementary to a region of A_2 and wherein this strand is the 'substrate' strand; and
    - the other stand comprises at least one quencher and wherein a region of this strand is complementary to a region of A_2 adjacent to that which the substrate strand is complementary to, such that in the presence of A_2 the partially double stranded nucleic acid construct has a greater double-stranded portion

22. A kit as claimed in claim 21, wherein the kit further comprises an enzyme for removal of the at least one RNA base.

23. A kit as claimed in any one of claims 1 to 12, wherein the kit 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.

24. A kit as claimed in any one of claims 1 to 23, wherein the kit further comprises a phosphatase or a phosphohydrolase.

25. A kit as claimed in any one of claims 1 to 24, wherein the kit further comprises a pyrophosphatase.

26. A kit as claimed in any one of claims 1 to 25, wherein the kit further comprises an enzyme for the formation of DNA from an RNA template.

~~27. A device comprising:~~

~~a fluid pathway between a first region and second region, wherein the first region comprises one or more wells, wherein one or more well comprises:~~

~~a single-stranded probe oligonucleotide A_0, which is capable of forming a first intermediate product with a target polynucleotide sequence, said intermediate product being at least partially double-stranded;~~

~~a pyrophosphorolysing enzyme capable of digesting the first intermediate product in the 3'-5' direction from the end of A_0 to create a partially digested strand A_1; a source of ions to drive the pyrophosphorolysis reaction forward, wherein optionally the ions are pyrophosphate ions;~~

~~and~~

~~one or more ligases capable of ligating A_1 to create an oligonucleotide A_2;~~

~~wherein the second region comprises one or more wells.~~

~~28. The device of claim 27, wherein the 5' end of A_0 is resistant to 5'-3' exonuclease digestion and wherein the wells of the first region further comprise a 5'-3' exonuclease.~~

~~29. The device of any one of claims 27 or 28, wherein the device further comprises a third region comprising one or more wells which is joined to the first region by a fluid pathway and wherein one or more wells of the third region comprises:~~

~~dNTPs;~~

~~at least one single-stranded primer oligonucleotide; and~~

~~an amplification enzyme.~~

~~30. The device of claim 29 wherein:~~

~~the dNTPs of the third region are dUTP, dGTP, dCTP and dATP;~~

~~the amplification enzyme is a dUTP incorporating high fidelity polymerase; and~~

~~one or more wells of the third region further comprises uracil-DNA N-glycosylase.~~

31. The device of claim 29 or claim 30 wherein the device further comprises a fourth region, located between the first and third regions, comprising one or more wells, wherein one or more wells comprises a proteinase.

32. The device of any one of claims 27 to 31, wherein one or more wells of the first or second regions further comprises a ligase and a ligation probe oligonucleotide C.

33. The device of any one of claims 27 to 31, wherein one or more wells of the first or second regions further comprises a ligase and a splint oligonucleotide D which is complementary to a region of A_0.

34. The device of any one of claims 27 to 31, wherein one or more wells of the first or second region further comprises a ligase, a splint oligonucleotide D and a ligation probe oligonucleotide C.

35. The device of claim 32 or claim 34, wherein the ligation probe oligonucleotide C comprises a 3' or internal modification protecting it from 3'-5' exonuclease digestion.

36. The device of any one of claims 33, 34 or 35, 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.

37. The device of any one of claims 27 to 36, wherein one or more wells of the first region comprises at least one or more different A_0, each selective for a different target sequence.

38. The device of claim 37, wherein the wells of the second region comprise:

dNTPs;

buffers;

an amplification enzyme;

one or more oligonucleotide binding dyes or molecular probes; and

a means for detecting a signal derived from A_1 or a portion thereof, or multiple copies of A_1 or multiple copies of a portion thereof.

39. The device of any one of claims 27 to 36, wherein the wells of the second region further comprise:

- two or more Ligation Chain Reaction (LCR) probe oligonucleotides that are complementary to adjacent sequences on A_1 wherein when the probes are successfully annealed the 5' phosphate of one LCR probe is directly adjacent to the 3'OH of the other LCR probe; and
- one or more ligases.

40. The device of any one of claims 27 to 36, wherein the wells of the second region further comprise:

- A ligation probe oligonucleotide C;
- A splint oligonucleotide D;

wherein C has a 5' phosphate, the 3' end of a splint oligonucleotide D is complementary to the 5' end of C and the 5' end of D is complementary to the 3' end of A_1 such that A_1 and C are capable of being ligated together to form an oligonucleotide A_2.

41. The device of claim 40, wherein the wells of the second region further comprise:

- A hairpin oligonucleotide 1 (HO1) comprising a fluorophore-quencher pair, wherein HO1 is complementary to A_2 and when annealed to A_2 the hairpin structure of HO1 opens and the fluorophore-quencher pair separate; and
- A hairpin oligonucleotide 2 (HO2) comprising a fluorophore-quencher pair, wherein HO2 is complementary to the open HO1 and when annealed to HO1 the hairpin structure of HO2 opens and the fluorophore-quencher pair separate.

42. The device of any one of claims 27 to 36, wherein the wells of the second region further comprise:

- an oligonucleotide A comprising a substrate arm, a partial catalytic core and a sensor arm;
- an oligonucleotide B comprising a substrate arm, a partial catalytic core and a sensor arm; and
- a substrate comprising a fluorophore-quencher pair;

wherein the sensor arms of oligonucleotides A and B are complementary to flanking regions of A_2 such that in the presence of A_2,oligonucleotides A and B are combined to form a catalytically, multicomponent nucleic acid enzyme (MNAzyme).

43. The device of any one of claims 27 to 36, wherein the wells of the second region further comprise a partially double-stranded nucleic acid construct wherein:

- one strand comprises at least one RNA base, at least one fluorophore and wherein a region of this strand is complementary to a region of A_2 and wherein this strand may be referred to as the 'substrate' strand;
- the other stand comprises at least one quencher and wherein a region of this strand is complementary to a region of A_2 adjacent to that which the substrate strand is complementary to, such that in the presence of A_2 the partially stranded nucleic acid construct becomes substantially more double-stranded; and

wherein the wells of the second region further comprise an enzyme for removal of the at least one RNA base.

44. The device of any one of claims 27 to 36 wherein one or more wells of the second region 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.

45. A device as claimed in any one of claims 27 to 44 wherein one or more wells of one or more regions further comprises a pyrophosphatase.

46. A device as claimed in any one of claims 27 to 45 wherein one or more wells of one or more regions further comprises a phosphatase or a phosphohydrolase.

47. A device as claimed in any one of claims 27 to 46 wherein one or more wells of the first region further comprises an enzyme for the formation of DNA from an RNA template.

~~48. A device as claimed in any one of claims 27 to 47 wherein the first and second regions are combined.~~