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(71) Applicant: HUBRO THERAPEUTICS AS [NO/NO];
Ullernchausséen 64, 0379 Oslo (NO).

(72) Inventors: ERIKSEN, Henrik; Ullernchausséen 64, 0379
Oslo (NO). ERIKSEN, Jon Amund; Camilla Collets vei
18, 0258 Oslo (NO).

(74) Agent: ARENDS, William Gerrit; 15 Fetter Lane, Lon-
don, Greater London EC4A 1BW (GB).

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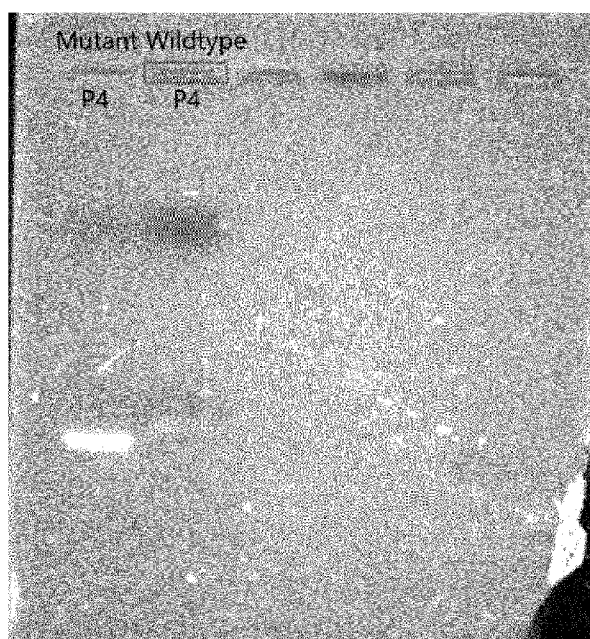
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Fig. 3



(57) Abstract: A primer for detection of a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule, wherein the mutation is a frameshift of the microsatellite as compared with the corresponding wild type microsatellite sequence. The primer comprises a region of at least 10 nucleotides that is complementary to the target sequence of the antisense or the sense strand of the DNA molecule containing the microsatellite having a frameshift mutation, except that the primer includes between one and four, or one and three, nucleotides which are mismatched to the target sequence containing the mutation in the microsatellite and which are also mismatched to a corresponding sequence containing the wild type microsatellite.



A Primer

Field of Invention

The present invention relates to a primer and, more specifically, a primer for detection of a mutation in a polynucleotide sequence. The present invention also relates to a kit comprising the primer and a method of detecting a mutation in a microsatellite sequence.

Background of Invention

Frameshift mutations can cause disease by disrupting normal protein translation. Amongst other pathologies, frameshift mutations have been linked with cancer, and in particular cancers that are linked to microsatellite instability. Microsatellite instability (MSI) is a hypermutable state of cells caused by an impairment in DNA mismatch repair (MMR). In other words, cells affected by MSI do not have proper functioning repair mechanisms and therefore accrue spontaneous mutations during DNA replications, which can cause frameshift mutations. These errors accumulate particularly in microsatellites, which are repeated sequences of DNA (most commonly a dinucleotide repeat of the nucleotides C and A). Therefore, MSI results in insertion and deletion mutations in these repeated sequences called microsatellites.

MSI has been linked to many cancers, including colon, gastric, endometrium, ovarian, hepatobiliary tract, urinary tract, brain and skin cancers (Cortes-Ciriano et al. 2017). Frameshift mutations have been found in ASTE1, TAF1B, KIAA2018 and SLC22A9. In particular, frameshift mutation of TGFBR2 is present in large numbers of colorectal cancers (CRC) and gastric cancers (GC) caused by microsatellite instability (MSI). Frameshift mutation of TGFBR2 is also known to be associated with Lynch Syndrome. MSI consists of insertion and deletion mutations in stretches of short tandem DNA repeats (microsatellites) throughout the genome. Over 95% of the frameshift mutations in CRC are reported to be single nucleotide deletions (Maby et al. 2015).

In view of the above, frameshift mutations offer a therapeutic target for treatment of diseases associated with such mutations, including many cancers. However, not all patients having such a disease will have this mutation, particularly for cancer patients where the disease is known to be highly heterogeneous. Thus, it is important to be able to detect the subset of patients within a disease population that have a frameshift mutation, in order to find the patients who would respond to therapy targeting the frameshift mutation.

For example, the cancer vaccine FMPV-1, which consists of a mutant immunogenic peptide, targets frameshift mutant TGFBR2 as described in WO/2020239937. Not all patients with MSI-

CRC (approximately 75%) and MSI-GC (approximately 80%) have frameshift mutated TGFBR2. In order to ensure recruitment of only eligible patients to clinical studies with FMPV-1 it would be highly useful to screen patients for detection of such a mutant TGFBR2 to find the patients who will benefit from such therapy. In other words, the detection of frameshift mutations may help to provide a personalised medicine approach for diseases where this mutation may or may not be present. In addition, detecting such mutations can assist with recruiting suitable candidates to a clinical trial for testing therapies that target such frameshift mutations.

This screening and personalised medicine approach would also be useful for patients with a single nucleotide deletion in the ASTE1 gene. In particular, the cancer vaccine FMPV-2 (also referred to as "fsp8") is a mutant immunogenic peptide targeting frameshift mutant ASTE1, as described in WO2021/239980. A single nucleotide deletion in this gene is the most dominant frameshift mutation, but does not occur in all MSI-H cancers. Therefore, it would be useful to screen patients for detection of such mutant ASTE1, to find the patients who will benefit from such therapy, as mentioned above in respect of TGF β R2.

Currently, it is necessary either to sequence the genome of the patient to understand their TGFBR2 and ASTE1 status, or to rely on DNA amplification followed by sequencing to detect the presence or absence of the frameshift mutation. These sequencing methods take around 2 days to complete and typically must be conducted by an external laboratory, as hospitals generally do not have the equipment available to carrying out such sequencing. This therefore requires samples to be transported to such a testing lab. . Therefore, there is a need to provide an easier assay for specific detection of frameshift mutations, particularly for the purposes of personalised medicine.

Polymerase chain reaction (PCR) is a well-established technology for analysing DNA sequences. The DNA specificity of a PCR test is determined by the primers designed to interact with the target DNA sequence. However, PCR primers cannot normally target a region with more than four nucleotide repeats, meaning the use of primers to detect a change in a microsatellite region is not trivial.

Thus, there is a need to develop a technique for specific detection in tumour biopsies of frameshift mutations that may be relevant in disease, in particular in TGFBR2 and ASTE1. Solid tumour biopsies are not representative of the entire tumour, since tumours tends to be heterogeneous and as such tumour biopsies are representative only of the part of tumour that the biopsy is taken from. In contrast, liquid biopsies are much more representative of whole cancer, and are easier

to work on. It is therefore desirable for this technique to be suitable for detection of cell free DNA (cfDNA) in liquid biopsies (e.g. plasma).

Summary of Invention

5 The present invention solves the needs and objectives above through the design of primers, and DNA amplification assays using such primers, that are able to distinguish between a microsatellite with a frameshift mutation, and a microsatellite with no frameshift mutation (i.e. a wild type microsatellite). In particular, these primers allow for the use of PCR to distinguish between a frameshift mutation and a wild type microsatellite even though they differ by only as little as a
10 single nucleotide, by for example, conducting the PCR under suboptimal conditions. These suboptimal conditions increase the stringency of the reaction so that the difference between the amplification efficiency between the frameshift mutant and the wild type microsatellite is magnified, providing a more sensitive test for the frameshift mutant microsatellite. These suboptimal conditions can be achieved in a number of different ways. Therefore, this is a useful
15 invention in the detection of frameshift mutations, diagnosis of MSI-related disease, and/or the screening of subjects for suitability for treatment against such frameshift mutations.

In one aspect of the invention, there is provided a primer for detection of a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule, wherein the
20 mutation is a frameshift of the microsatellite as compared with the corresponding wild type microsatellite sequence, and wherein the primer comprises a region of at least 10 nucleotides that is complementary to the target sequence of the antisense or the sense strand of the DNA molecule containing the microsatellite having a frameshift mutation, except that the primer includes between one and four, or between one and three, nucleotides which are mismatched to
25 the target sequence containing the mutation in the microsatellite and which are also mismatched to a corresponding sequence containing the wild type microsatellite. In some embodiments, the primer includes between one and three nucleotides which are mismatched to the target sequence containing the mutation in the microsatellite and which are also mismatched to a corresponding sequence containing the wild type microsatellite.

30 In some embodiments, the primer is configured to anneal across the length of the microsatellite having a frameshift mutation, and to at least one nucleotide, or at least two nucleotides, flanking the 5' and/or at least one nucleotide flanking the 3' end of the microsatellite having a frameshift mutation. In some embodiments, the primer is configured to anneal across the length of the
35 microsatellite having a frameshift mutation, and to at least two nucleotides flanking the 5' and/or at least two nucleotides flanking the 3' end of the microsatellite having a frameshift mutation.

In some embodiments, the primer consists of between 16 and 30 nucleotides.

In some embodiments, at least one mismatched nucleotide is located in a position of the primer
5 that is configured to anneal 3' downstream of the microsatellite or within the microsatellite.

In some embodiments, at least one mismatched nucleotide is within three or four nucleotides 5' upstream or 3' downstream of the 3' end of the microsatellite.

10 In some embodiments, at least one mismatched nucleotide is a substitution of a thymine (T) with an adenine (A), a substitution of an adenine (A) with a guanine (G), a substitution of a thymine (T) with a cytosine (C) or a substitution of a cytosine (C) with a guanine (G).

15 In another aspect of the invention there is provided a kit for detection of a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule, wherein the mutation is a frameshift of the microsatellite as compared with the corresponding wild type microsatellite sequence, and wherein the kit comprises a first primer of the invention, and a second primer, wherein the second primer is configured to anneal to the target sequence 3' downstream of the microsatellite on the opposite strand of the DNA molecule to the strand on
20 which the first primer is configured to anneal.

It is to be understood that where reference is made to "3' downstream" this is with respect to the (first) primer, i.e. in the direction of DNA polymerase extension of the (first) primer. In other words, the term "3' downstream" refers to a position 3' in the primer, which corresponds to a position 5' in the template sequence. For example, the expression "at least one mismatched nucleotide is
25 located in a position of the primer that is configured to anneal 3' downstream of the microsatellite", above, means that the at least one mismatched nucleotide is 3' downstream, in the primer, of the microsatellite, i.e. 5' upstream of the microsatellite in the template sequence. Similarly, it is to be understood that where reference is made to the "3' end of the microsatellite having a frameshift mutation", this is with respect to the (first) primer. In other words, the term "3' end" refers to the 3' end in the primer, which corresponds to the 5' end in the template sequence.
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Similarly, it is to be understood that, where reference is made to "5' upstream", this is with respect to the (first) primer. For example, the expression "the primer is configured to anneal across the
35 length of the microsatellite having a frameshift mutation, and to at least two nucleotides flanking the 5' and/or at least two nucleotides flanking the 3' end of the microsatellite having a frameshift

mutation", above, means that the primer may anneal to at least two nucleotides flanking the 5' end of the microsatellite with respect to the primer, i.e. to at least two nucleotides flanking the 3' end of the microsatellite with respect to the template. Similarly, it is to be understood that where reference is made to the "5' end of the microsatellite having a frameshift mutation", this is with respect to the (first) primer. In other words, the term "5' end" refers to the 5' end in the primer, which corresponds to the 3' end in the template sequence.

In some embodiments, the frameshift mutation is in a microsatellite in the TGFBR2 gene and comprises the sequence according to residues 270 to 278 of SEQ ID NO: 20, and wherein the first primer comprises the sequence defined by any one of SEQ ID NO: 3, 4, 5 or 7, and/or the second primer comprises the sequence defined by SEQ ID NO: 2.

In some embodiments, the frameshift mutation is in a microsatellite in the ASTE1 gene and comprises the sequence according to residues 328-337 of SEQ ID NO: 32, and wherein the first primer comprises the sequence defined by SEQ ID NO: 15 or 31, and/or the second primer comprises the sequence defined by SEQ ID NO: 10.

In some embodiments, the kit further comprises:

a third primer, wherein the third primer is configured to anneal 5' upstream of the region to which the second primer is configured to anneal, and wherein the third primer is configured to anneal to the same strand as the first primer and the opposite strand to the second primer, or another control primer pair.

In some embodiments, the sequence containing the wild type microsatellite comprises the sequence defined in SEQ ID NO: 19, and preferably the third primer comprises the sequence defined by SEQ ID NO: 1.

In some embodiments, the sequence containing the wild type microsatellite comprises the sequence defined in SEQ ID NO: 32, and preferably the third primer comprises the sequence defined in SEQ ID NO: 11 or SEQ ID NO: 38.

In some embodiments, the kit further comprises the components for carrying out DNA amplification, preferably wherein the components comprise at least one of: a buffer, dNTPs, and Taq-polymerase.

In another aspect of the invention, there is provided a primer for DNA amplification comprising the sequence defined by any one of SEQ ID NO: 3, 4, 5, 7, 15, 31, 39, 40 or 41, preferably one of SEQ ID NOs: 3, 15, 31, 39, 40 or 41, more preferably one of SEQ ID NOs: 3, 15 and 31.

5 In another aspect of the invention there is provided a method for detecting a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule, wherein the mutation is a frameshift of the microsatellite as compared with the corresponding wild type microsatellite sequence, and the method comprising:

a) providing a first aliquot of a sample comprising human DNA,

10 b) adding to the first aliquot the necessary components for DNA amplification, a first primer and a second primer; wherein the first primer is suitable for detection of a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule and comprises a region of nucleotides that is complementary to the target sequence containing the microsatellite having a frameshift mutation except for between one and four, or one
15 and three, nucleotides which are mismatched to the target sequence containing the microsatellite having a frameshift mutation and which are also mismatched to a corresponding sequence containing the wild type microsatellite and wherein the first primer is configured to anneal across the length of the microsatellite having a frameshift mutation and to anneal to at least one nucleotide flanking the 3' end of the microsatellite
20 having a frameshift mutation and at least one nucleotide flanking the 5' end of the microsatellite having a frameshift mutation; and the second primer is configured to anneal to the target sequence 3' downstream of the microsatellite, to form a first reaction mix; wherein the first primer is configured to anneal to the sense strand of the DNA molecule and the second primer is configured to anneal to the antisense strand of the DNA
25 molecule, or the first primer is configured to anneal to the antisense strand of the DNA molecule and the second primer is configured to anneal to the sense strand of the DNA molecule,

c) carrying out DNA amplification on the first reaction mix,

d) detecting the presence of the frameshift mutation in the sample when an amplification
30 product is produced from the DNA amplification of the first reaction mix. In some embodiments, the primer comprises between one and three nucleotides which are mismatched to the target sequence containing the microsatellite having a frameshift mutation and which are also mismatched to a corresponding sequence containing the wild type microsatellite.

35 In some embodiments, the method further comprises:

a) also adding to the first aliquot either:

I) the second primer and a third primer, wherein the third primer is configured to anneal 5' upstream of the region to which the second primer is configured to anneal, and wherein the third primer is configured to anneal to the same strand as the first primer and the opposite strand to the second primer or

II) a control primer pair, to form the first reaction mix or

b) providing a second aliquot of the sample comprising human DNA and adding to the second aliquot the necessary components for DNA amplification and either:

I) the second primer and a third primer, wherein the third primer is configured to anneal 5' upstream of the region to which the second primer is configured to anneal, and wherein the third primer is configured to anneal to the same strand as the first primer and the opposite strand to the second primer or

II) a control primer pair, to form a second reaction mix

carrying out DNA amplification on the reaction mix, and detecting that DNA amplification has been carried out successfully when an amplification product is produced from the DNA amplification of the second reaction mix.

In some embodiments, DNA amplification is polymerase chain reaction (PCR), wherein the PCR comprises a plurality of cycles of denaturation, annealing and extension.

In some embodiments, the reaction mix(es) comprises 1x or 0.5x buffer, 0.4mM dNTPs, 0.2μM forward primer, and 0.2μM reverse primer. In some embodiments, the reaction mix(es) comprises a final concentration of dNTPs of 200μM. In some embodiments, the reaction mix(es) comprises, as a final concentration, 1x or 0.5x buffer, 200μM dNTPs, 0.2μM forward primer and 0.2μM reverse primer.

In some embodiments, step d) further comprises running the product of the PCR reaction on a gel and visualising a band to confirm that DNA amplification has been successful.

In some embodiments, the method further comprises step f) cutting out the band for DNA sequencing.

In some embodiments, the frameshift mutation is in a microsatellite in the TGFBR2 gene and the target sequence comprises the sequence according to residues 270 to 278 of SEQ ID NO: 20, and wherein the first primer comprises the sequence defined by any one of SEQ ID NO: 3, 4, 5, 7 or 39 and/or the second primer comprises the sequence defined by SEQ ID NO: 2; or the

frameshift mutation is in a microsatellite in the ASTE1 gene and the target sequence comprises the sequence according to residues 328-337 of SEQ ID NO: 33, and wherein the first primer comprises the sequence defined by SEQ ID NO: 15, 31, 40 or 41, and/or the second primer comprises the sequence defined by SEQ ID NO: 10.

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In some embodiments, the microsatellite is in a TGFBR2 gene, the sequence comprising the wild type microsatellite consists of the sequence according to SEQ ID NO: 19, and wherein the third primer comprises the sequence defined by SEQ ID NO: 1; or the microsatellite is in an ASTE1 gene, the sequence comprising the wild type microsatellite comprises the sequence according to SEQ ID NO: 32, and wherein the third primer comprises the sequence defined by SEQ ID NO: 11 or SEQ ID NO: 38.

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In another aspect of the invention, there is provided a method of diagnosing a disease associated with a frameshift mutation in a microsatellite, comprising carrying out the method for detecting a mutation in a microsatellite according to the invention.

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In some embodiments, the method further comprises determining that a patient suffering from a disease or disorder associated with a frameshift mutation is suitable for a treatment targeting said frameshift mutation if the frameshift mutation is detected in step d) in a sample from the patient.

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In some embodiments, the disease or disorder associated with a frameshift mutation is a cancer.

In some embodiments, the disease or disorder is colorectal cancer or gastric cancer, and the treatment targeting the frameshift mutation is FMPV-1, wherein the frameshift mutation is in a microsatellite in the TGFBR2 gene and comprises the sequence according to residues 270 to 278 of SEQ ID NO: 20; or the disease or disorder is endometrial cancer or gastric cancer and the treatment targeting the frameshift mutation is FMPV-2, and the frameshift mutation is in a microsatellite in the ASTE1 gene and comprises the sequence according to residues 328 to 337 of SEQ ID NO: 33.

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In some embodiments, the method further comprises step e) of treating the patient with FMPV-1 or FMPV-2.

In some embodiments, the microsatellite is in a TGFBR2 gene, the sequence comprising the wild type microsatellite comprises the sequence according to SEQ ID NO: 19, and the third primer comprises the sequence defined by SEQ ID NO: 1.

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In some embodiments, the microsatellite is in an ASTE1 gene, the sequence comprising the wild type microsatellite comprises the sequence according to SEQ ID NO: 32, and the third primer comprises the sequence defined by SEQ ID NO: 11 or SEQ ID NO: 38.

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In some embodiments, the first primer comprises the sequence defined by any one of SEQ ID NO: 3, 4, 5, 7 or 39, and/or the second primer comprises the sequence defined by SEQ ID NO: 2; or the first primer comprises the sequence defined by SEQ ID NO: 15, 31, 40 or 41, and/or the second primer comprises the sequence defined by SEQ ID NO: 10.

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In some embodiments, the sample is a liquid biopsy comprising cell free DNA, preferably wherein the liquid biopsy is plasma.

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In some embodiments, the DNA amplification is PCR, and the PCR is carried out in high stringency conditions, optionally wherein the high stringency conditions comprise at least one of:

a) carrying out the annealing step of PCR at a temperature that is at least 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C or 10°C higher than the recommended annealing temperature of the reaction;

b) carrying out the annealing step of PCR for only 30 seconds, preferably 15 seconds, per cycle;

20

c) carrying out the DNA amplification in a buffer concentration that is less than 0.1X, 0.2X, 0.3X, 0.4X, 0.5X, 0.6X, or 0.75X;

d) carrying out the DNA amplification in a buffer comprising ammonium ions; and

e) performing 25, 20, 15, or 10 cycles of PCR, or fewer than 25, fewer than 20, fewer than 15 or fewer than 10 cycles of PCR.

25

In some embodiments, the buffer includes ammonium (NH₄⁺) ions.

In some embodiments, the buffer includes ammonium (NH₄⁺) ions and is at a concentration of 1X.

30

In some embodiments, the buffer is 1X Key buffer.

Definitions

In this specification, the following terms may be understood as follows:

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The terms “gene”, “polynucleotides”, and “nucleic acid molecules” are used interchangeably herein to refer to a polymer of multiple nucleotides. The nucleic acid molecules may comprise

naturally occurring nucleic acids (i.e. DNA or RNA) or may comprise artificial nucleic acids such as peptide nucleic acids, morpholin and locked nucleic acids as well as glycol nucleic acids and threose nucleic acids.

- 5 The term “nucleotide” as used herein refers to naturally occurring nucleotides and synthetic nucleotide analogues that are recognised by cellular enzymes.

The phrase “high stringency conditions” means conditions that only allow for successful DNA amplification where there is a very high level of matching to the target sequence. In other words, the conditions are suboptimal for DNA amplification in order that primers with lower sequence matching to the target sequence are not able to anneal. Such suboptimal conditions can be generated by altering one or more of temperature, cycle number, ionic strength and the presence of certain organic solvents that allow pairing of nucleic acid sequences. Further details on the high stringency conditions are provided below. In particular, the high stringency conditions may include where the annealing step of PCR is carried out at a temperature that is at least 2%, 5%, or 10% higher than the recommended annealing temperature of the reaction. Alternatively, the annealing step of PCR is carried out at a temperature that is at least 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C or 10°C higher than the recommended annealing temperature of the reaction. In some embodiments, the annealing step of PCR is carried out at a temperature that is 4°C higher than the recommended annealing temperature of the reaction. For example, the annealing step may be carried out between 44°C and 61°C or 62°C, as further detailed below. The annealing step may additionally or alternatively be carried out for a shorter period than is recommended for the reaction, for example, the annealing step may be reduced from 45 seconds to 30 seconds or 15 seconds. Additionally or alternatively, the high stringency conditions may include using a lower buffer concentration than is recommended for the reaction, such as 10%, 20%, 30%, 40%, 50%, 60% or 75% of the recommended concentration of a buffer in a reaction, in particular the buffer concentration may be less than 0.1X, 0.2X, 0.3X, 0.4X, 0.5X, 0.6X or 0.75X. Additionally or alternatively, the high stringency conditions may include using a more stringent buffer in the reaction, such as a buffer including ammonium ions (NH_4^+), for example, Key buffer. A more stringent buffer may be used at 1X concentration. In some embodiments, 1X Key buffer is used. Additionally or alternatively, the high stringency conditions may include reducing the number of cycles in a PCR, such as carrying out 25 cycles, 20 cycles, 15 cycles or even 10 cycles, compared to 30, 35 or 40 cycles. In some embodiments, 30 or fewer, 25 or fewer, 20 or fewer, 15 or fewer or 10 or fewer cycles of PCR are carried out. Any one or more of the above conditions may be used.

The term “frameshift” means a genetic mutation caused by a deletion or insertion in a DNA sequence that causes a shift in the sequence, meaning that the nucleotides are grouped into a different series of codons resulting in a different protein being translated from this sequence. In many cases, the frameshift may cause a premature stop codon in the protein sequence, resulting in a truncated protein sequence. For example, one such frameshift mutation in TGFBR2 is the deletion of a single adenine, meaning that a sequence of 10 adenines (a10; which is a microsatellite) is reduced to 9 adenines (a9). The sequence of wild type TGFBR2 is shown in SEQ ID NO: 34 and the sequence of a9 TGFBR2 is shown in SEQ ID NO: 35. Another such frameshift is a deletion of a single adenine in ASTE1, meaning that a sequence of 11 adenines (a11; which is a microsatellite) is reduced to 10 adenines (a10). The sequence of wild type ASTE1 is shown in SEQ ID NO: 36 and the sequence of a10 ASTE1 is shown in SEQ ID NO: 37.

The term “microsatellite” means a region of DNA where a sequence is repeated, typically between 5 and 50 times. Microsatellites may also be particularly vulnerable to mutation. Microsatellites may also be known as “short tandem repeats (STRs)”. Microsatellites may be a repeated series of a single nucleotide such as A, G, C or T, or may be a repeated series of a longer motif, such as TA (dinucleotide repeat) or GTC (trinucleotide repeat).

As is well known in molecular biology, guanine (G) and cytosine (C), and adenine (A) and thymine (T), are complementary nucleotides, and thus will pair e.g. during DNA replication. In the current invention, a “mismatch” or a “mismatched” nucleotide may be a deletion of a complementary nucleotide, an addition of a nucleotide, or a substitution of a complementary nucleotide for a non-complementary (i.e. mismatched) nucleotide. For example, a mismatch to a target guanine (G) may be an adenine (A), a thymine (T) or another guanine (G), but not a cytosine (C). In particular, where a frameshift mutation causes an increase in the microsatellite length, then an addition of a nucleotide is particularly useful for distinguishing the frameshift mutated microsatellite from the wild type microsatellite. In contrast, where a frameshift mutation causes a decrease in the microsatellite length, then a deletion of a nucleotide is particularly useful for distinguishing the frameshift mutated microsatellite from the wild type microsatellite. In one example, this deletion is a deletion of a single nucleotide from a microsatellite sequence.

Again, as is well known in the field of molecular biology, in humans DNA is formed of a double stranded DNA helix formed of two strands, a sense and an antisense strand. Thus the term “sense strand” will be known to the skilled person as the coding strand, carrying transcribable nucleotides in a 5' to 3' direction and the term “antisense strand” will be known to the skilled person as a

strand having a reverse complementary sequence to the coding strand in a 5' to 3' direction, and being the template for mRNA transcription.

The term “primer” refers to a short single stranded DNA sequence that is used to initiate targeted DNA amplification. It is typically between 18 and 24 bases in length, but is shorter or longer than this typical length in some embodiments. A “primer pair” is two such primers that cause DNA amplification of a specific target sequence lying between the annealing sites of these two primers.

The term “treating” refers to any partial or complete treatment and includes: inhibiting the disease or symptom, i.e. arresting its development; and relieving the disease or symptom, i.e. causing regression of the disease or symptom.

Brief Description of the Figures

Figure 1 shows two electrophoresis gels of the products of PCR reactions carried out on 10a microsatellite TGFBR2 template DNA (i.e. wild type) using primer pair P1 and P2 using different quantities of template DNA. Figure 1A shows results with increasing amounts of template DNA in nanograms (ng), and Figure 1B shows results with increasing amounts of template DNA in pictograms (pg).

Figure 2 shows an electrophoresis gel of the products of PCR reactions carried out on 9a microsatellite TGFBR2 template DNA (i.e. mutant) and 10a microsatellite TGFBR2 template DNA (i.e. wild type) for primers P1 and P4 in combination with primer P2, at a more stringent condition using an annealing temperature of 53°C and a reduced cycle number (25).

Figure 3 shows an electrophoresis gel of the products of PCR reactions carried out on 9a microsatellite TGFBR2 template DNA (i.e. mutant) and 10a microsatellite TGFBR2 template DNA (i.e. wild type) for primer pair P4 and P2 at an even more stringent condition using an annealing temperature of 55°C.

Figure 4 shows an electrophoresis gel of the products of PCR reactions carried out on 9a microsatellite TGFBR2 template DNA (i.e. mutant) and 10a microsatellite TGFBR2 template DNA (i.e. wild type) for primers P6 and P10 in combination with primer P2. In particular, those lanes denoted with an asterisk (*) were those for products of a PCR reaction carried out at a low buffer concentration.

Figure 5 shows three electrophoresis gels of the products of PCR reactions carried out on 9a microsatellite TGFBR2 template DNA (i.e. mutant) for primers P4, P6 and P10 in combination with primer P2 (as well as primer P1 in combination with primer P2 as a positive control) at increasingly stringent PCR conditions. The reactions in Figure 5A were carried out at an annealing temperature of 55°C for 30 cycles. The reactions in Figure 5B were carried out at an annealing temperature of 56°C for 25 cycles. The reactions in Figure 5C were carried out at an annealing temperature of 58°C for 25 cycles. Again, those lanes denoted with an asterisk (*) were those for products of a PCR reaction carried out at a low buffer concentration.

Figure 6 shows two electrophoresis gels of the products of PCR reactions carried out on 9a microsatellite TGFBR2 template DNA (i.e. mutant) and 10a microsatellite TGFBR2 template DNA (i.e. wild type) for primers P1, P6 and P10 in combination with primer P2. In particular, those lanes denoted with an asterisk (*) were those for products of a PCR reaction carried out at a low buffer concentration. The reactions in Figure 6A were carried out with primer pairs and the reactions in Figure 6B were carried out with three primers.

Figure 7 shows an electrophoresis gel of the products of PCR reactions carried out on 9a microsatellite TGFBR2 template DNA (i.e. mutant) and 10a microsatellite TGFBR2 template DNA (i.e. wild type) for primers P1, P4 and P10 in combination with primer P2. This includes three primer reactions that include both primers P1 and P2 as well primer P4 or P10.

Figure 8 shows an electrophoresis gel of the products of PCR reactions carried out on 9a microsatellite TGFBR2 template DNA (i.e. mutant) and 10a TGFBR2 microsatellite template DNA (i.e. wild type) for each of the primer combinations listed, demonstrating that all of these primers are functional.

Figure 9 shows two electrophoresis gels of the products of PCR reactions carried out on 9a microsatellite TGFBR2 template DNA (i.e. mutant) DNA for primers P1, P4.1, P4.2 and P10.1, where the PCR reactions have either been carried out in standard Taq (KCl) buffer or Key buffer (containing ammonium ions, NH_4^+). The reactions in Figure 9A were carried out at an annealing temperature of 56°C and the reactions in Figure 9B were carried out at an annealing temperature of 58°C.

Figure 10 shows a gel electrophoresis of the PCR reactions carried out on 9a microsatellite template TGFBR2 DNA (i.e. mutant) and 10a microsatellite TGFBR2 template DNA (i.e. wild type) for primers P4, P4.1, P4.2 and P6 in combination with primer P2. The reactions in Figure 10A

were carried out using standard Taq buffer, and the reactions in Figure 10B were carried out using Key buffer (comprising ammonium, NH_4^+).

Figure 11 shows a gel electrophoresis of the PCR reactions carried out on 9a microsatellite TGFB2 template DNA (i.e. mutant) and 10a microsatellite TGFB2 template DNA (i.e. wild type) for primers P4, P4.1, P4.2 and P6 in combination with primer P2. The reactions in Figure 11A were carried out using standard Taq buffer (which does not comprise ammonium), and the reactions in Figure 11B were carried out using Key buffer (comprising ammonium, NH_4^+).

Figure 12 shows the results of the detection of TGFB2 cfDNA from MSI-CRC and microsatellite stable-CRC (MSS-CRC) patients using primers defining a fragment of TGFB2 comprising the a10/a9 microsatellite. ¹primer designed for detection of TGFB2 a9 (mutant) microsatellite; ²Control primers for detection of both a9 and a10 TGFB2 microsatellites.

Figure 13 shows an electrophoresis gel of the products of PCR reactions carried out on 9a microsatellite TGFB2 template DNA (i.e. mutant, F1) and 10a microsatellite TGFB2 template DNA (i.e. wildtype, F2) using primers P2 and P4 at different annealing temperatures.

Figure 14 shows an electrophoresis gel of the products of PCR reactions carried out on (A) 9a microsatellite TGFB2 template DNA (i.e. mutant, F1) and (B) 10a microsatellite TGFB2 template DNA (i.e. wildtype, F2) using primers P2 and P4 at different annealing temperatures.

Figure 15 shows an electrophoresis gel of the products of PCR reactions carried out using primer P4 on 9a microsatellite TGFB2 template DNA (i.e. mutant, F1) and 10a microsatellite TGFB2 template DNA (i.e. wildtype, F2) using standard Taq (KCl) buffer using an annealing temperature of 57°C. Primer P2 was the forward primer for all reactions and primer P1 was the positive control reverse primer.

Figure 16 shows an electrophoresis gel of the products of PCR reactions carried out with primers P11 and P16 on (A) 10a microsatellite ASTE1 template DNA (i.e. mutant, F3) and (B) 11a microsatellite ASTE1 template DNA (i.e. wild type, F4), using a range of annealing temperatures.

Figure 17 shows electrophoresis gels of the products of PCR reactions carried out with primer P24 on (A) 10 microsatellite ASTE1 template DNA (i.e. mutant, F3, referred to as "M" in the gels) and (B) 11a microsatellite ASTE1 template DNA (i.e. wild type, F4, referred to as "Wt" in the gels), using a range of annealing temperatures.

Figure 18 shows electrophoresis gels of the products of PCR reactions carried out with primer P12 on (A) A) 10 microsatellite ASTE1 template DNA (i.e. mutant, F3, referred to as "M" in the gels) and (B) 11a microsatellite ASTE1 template DNA (i.e. wild type, F4, referred to as "Wt" in the gels), using a range of annealing temperatures.

Detailed Description of the Invention

In view of the above, there is a need to develop a tool for specific detection of frameshift mutations that may be relevant in disease, such as a frameshift mutation in the microsatellite of TGFBR2 or ASTE1. There is a further need for this tool to be suitable for detection of cell free DNA (cfDNA) in liquid biopsies (e.g. plasma).

Thus, in a first aspect of the invention there is provided a primer for detection of a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule, wherein the mutation is a frameshift of the microsatellite as compared with the corresponding wild type microsatellite sequence, and wherein the primer comprises a region of at least 10 nucleotides that is complementary to the target sequence of the antisense or the sense strand of the DNA molecule containing the microsatellite having a frameshift mutation, except that the primer includes between one and four, or between one and three, nucleotides which are mismatched to the target sequence containing the mutation in the microsatellite and which are also mismatched to a corresponding sequence containing the wild type microsatellite. In some embodiments, the primer includes between one and three nucleotides which are mismatched to the target sequence containing the mutation in the microsatellite and which are also mismatched to a corresponding sequence containing the wild type microsatellite.

As is disclosed herein, there are many microsatellites in which disease-relevant frameshift mutations can occur. For example, the frameshift may be in a microsatellite in any gene selected from ASTE1, ACVR22, TAF1B, KIAA2018, SLC22A9 and TGFBR2. In some embodiments, the frameshift is in a microsatellite in TGFBR2 or ASTE1.

In some embodiments, the primer is configured to anneal across the length of the microsatellite having a frameshift mutation, and to at least one nucleotide, or at least two nucleotides, flanking the 5' end of the microsatellite and/or at least one nucleotide flanking the 3' end of the microsatellite having a frameshift mutation. In some embodiments, the primer is configured to anneal across the length of the microsatellite having a frameshift mutation, and to at least two nucleotides flanking the 5' end of the microsatellite having a frameshift mutation and/or at least

two nucleotides flanking the 3' end of the microsatellite having a frameshift mutation. The primer may anneal to at least three, four, or five nucleotides flanking one or both ends of the microsatellite having a frameshift mutation.

5 It is to be understood that where reference is made to "anneal across the length of the microsatellite having a frameshift mutation", this means that the primer anneals to the target sequence at least for the full length of the microsatellite in the target sequence (which has a frameshift mutation). For example, when the target sequence is a9 TGFBR2, the primer anneals to the target sequence for the whole length of the a9 microsatellite. When the target sequence is
10 a10 ASTE1, the primer anneals to the target sequence for the whole length of the a10 microsatellite.

In some embodiments, the primer does not anneal across the length of the corresponding wild-type microsatellite sequence, and the 3' end of the primer does not anneal to the corresponding
15 sequence containing the wild-type microsatellite. This provides the advantage that the primer anneals to the sequence containing the microsatellite having a frameshift mutation, but does not anneal to the corresponding sequence having the wild-type microsatellite.

In some embodiments, the primer anneals to between 1 and 15, preferably between 1 and 13,
20 nucleotides flanking the 3' end of the microsatellite having a frameshift mutation (i.e. the primer comprises between 1 and 15, preferably between 1 and 13, nucleotides 3' of the microsatellite, with respect to the primer, which anneal to the corresponding sequence comprising the microsatellite having a frameshift mutation). In some embodiments, the primer anneals to one, two, three, five or thirteen, preferably one, two, or thirteen, nucleotides flanking the 3' end of the
25 microsatellite having a frameshift mutation (i.e. the primer comprises one, two, three, five or thirteen, preferably one, two or thirteen, nucleotides 3' of the microsatellite, with respect to the primer, which anneal to the corresponding sequence comprising the microsatellite having a frameshift mutation). In some embodiments, the primer anneals to two or three nucleotides flanking the 3' end of the microsatellite having a frameshift mutation and in some embodiments
30 these two or three nucleotides form a GC-clamp. In some embodiments, the primer anneals across the length of the microsatellite having the frameshift mutation and, 3' of the microsatellite having the frameshift mutation, with respect to the primer, the primer comprises or consists of two, three, five or thirteen, preferably one, two, five or thirteen, nucleotides which anneal to the nucleotides flanking the microsatellite having the frameshift mutation. This provides the
35 advantage that the primer annealed to the sequence containing the microsatellite having a frameshift mutation can be extended in the 5'-to-3' direction, thereby allowing replication of the

sequence containing the microsatellite having the frameshift mutation. In some embodiments, the primer anneals to one, two, three or five nucleotides flanking the 3' end of the microsatellite having a frameshift mutation and the frameshift mutation is in a microsatellite in TGFBR2. In some embodiments, the primer anneals to two, three or thirteen, preferably two or thirteen, nucleotides flanking the 3' end of the microsatellite having a frameshift mutation and the frameshift mutation is in a microsatellite in ASTE1.

In some embodiments, the primer anneals to at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 nucleotides flanking the 5' end of the microsatellite having a frameshift mutation, with respect to the primer. In some embodiments, the primer anneals to 1, 8 or 12 nucleotides flanking the 5' end of the microsatellite having a frameshift mutation, with respect to the primer. The primer may anneal to any of the above-detailed number of nucleotides flanking the 3' end of the microsatellite having a frameshift mutation.

In some embodiments, the primer is configured to anneal to at least 1, at least 2, at least 3, at least 5 or at least 12 nucleotides flanking the 5' end of the microsatellite having a frameshift mutation and to at least 2, at least 3 or at least 13 nucleotides flanking the 3' end of the microsatellite having a frameshift mutation. In some embodiments, the primer is configured to anneal to 1, 8 or 12 nucleotides flanking the 5' end of the microsatellite having a frameshift mutation and to anneal to least 2, at least 3 or at least 13, preferably to 2, 3, 5 or 13, nucleotides flanking the 3' end of the microsatellite having a frameshift mutation. In some embodiments, the primer is configured to anneal to 12 nucleotides flanking the 5' end of the microsatellite having a frameshift mutation and to 2 nucleotides flanking the 3' end of the microsatellite having a frameshift mutation, to 8 nucleotides flanking the 5' end of the microsatellite having a frameshift mutation and to 5 nucleotides flanking the 3' end of the microsatellite having a frameshift mutation, to 12 nucleotides flanking the 5' end of the microsatellite having a frameshift mutation and to 1 nucleotide flanking the 3' end of the microsatellite having a frameshift mutation, or to 1 nucleotide flanking the 5' end of the microsatellite having a frameshift mutation and to 13 nucleotides flanking the 3' end of the microsatellite having a frameshift mutation.

In some embodiments, at least 30%, 35%, 40%, 45%, 50%, 55%, 60% or 65% of the nucleotides of the primer anneal to nucleotides flanking the 5' end of the microsatellite having a frameshift mutation, with respect to the primer. In other words, at least 30%, 35%, 40%, 45%, 50%, 55%, 60% or 65% of the nucleotides making up the primer anneal to nucleotides flanking the 5' end of the microsatellite having a frameshift mutation with respect to the primer (i.e. flanking the 3' end of the microsatellite having a frameshift mutation with respect to the target sequence). In some

embodiments, the part of the primer which anneals to the nucleotides flanking the 5' end the microsatellite having a frameshift mutation, with respect to the primer, also anneals to the corresponding sequence containing the wild-type microsatellite. This provides the advantage that the primer is specific for the desired target sequence. In some embodiments, at least 50% of the nucleotides of the primer anneal to nucleotides flanking the 5' end of the microsatellite having a frameshift mutation with respect to the primer (i.e. anneal to nucleotides flanking the 3' end of the microsatellite having a frameshift mutation with respect to the target sequence).

In some embodiments, the nucleotide(s) of the primer which anneal to nucleotide(s) flanking the 5' end of the microsatellite having a frameshift mutation, with respect to the primer, are 100% complementary to the corresponding nucleotide(s) in the target sequence having the frameshift mutation in the microsatellite. This provides the advantage that the primer is specific to the target sequence.

In some embodiments, the primer consists of between 16 and 30 nucleotides. Preferably, the primer consists of between 16 and 25 nucleotides or between 16 and 24 nucleotides. Preferably still, the primer consists of between 17 and 24, or between 17 and 23 nucleotides.

In some embodiments, the primer is isolated or recombinant. In some embodiments, the primer is less than 50, 30, or 20 nucleotides in length.

In some embodiments, the primer consists of 22, 23 or 24 nucleotides.

In some embodiments, the wild type microsatellite sequence is a sequence from human genomic DNA.

In some embodiments, the primer comprises 1, 2, 3 or 4 nucleotides which are mismatched to the target sequence containing the mutation in the microsatellite and which are also mismatched to a corresponding sequence containing the wild type microsatellite. This means that the primer contains between 1 and 4 such mismatches, but no more than 4 such mismatches. In some embodiments, at least one mismatched nucleotide is located in a position of the primer that anneals 3' downstream of the microsatellite or within the microsatellite. In some embodiments, at least one mismatched nucleotide is located in a position of the primer that anneals 3' downstream of the microsatellite and at least one mismatched nucleotide is located within the microsatellite, with respect to the primer. In some embodiments, the primer has only one mismatched nucleotide, which is located in a position of the primer that anneals 3' downstream of the microsatellite. In

some embodiments, the primer has one mismatched nucleotide located in a position of the primer that anneals 3' downstream of the microsatellite and two mismatched nucleotides located within the microsatellite. In some embodiments, the primer has one mismatched nucleotide located in a position of the primer that anneals 3' downstream of the microsatellite and one mismatched nucleotide located within the microsatellite, with respect to the primer. In some embodiments, the primer has three mismatched nucleotides located within the microsatellite, with respect to the primer. In some embodiments, the primer has one mismatched nucleotide located within the microsatellite and two mismatched nucleotides located in a position of the primer that anneals 3' downstream of the microsatellite, with respect to the primer. In some embodiments, all of the mismatched nucleotides are located in a position of the primer that anneals 3' downstream of the microsatellite or within the microsatellite. In some embodiments, all of the mismatched nucleotides are located in a position of the primer that anneals within the microsatellite.

In some embodiments, at least one mismatched nucleotide is within three nucleotides 5' upstream or 3' downstream of the 3' end of the microsatellite with respect to the primer. In some embodiments, at least one mismatched nucleotide is within five nucleotides 3' downstream of the 3' end of the microsatellite, with respect to the primer. Preferably, all of the mismatched nucleotides are within three nucleotides 5' upstream or 3' downstream of the 3' end of the microsatellite with respect to the primer.

Each of the mismatches in the primer may be a nucleotide substitution to any nucleotide (e.g. A, T, C or G) which is mismatched to the corresponding nucleotide in the target sequence and the wild type sequence. In some embodiments, at least one mismatched nucleotide is a substitution of a thymine (T) with an adenine (A), a substitution of an adenine (A) with a guanine (G), a substitution of a thymine (T) with a cytosine (C), or a substitution of a cytosine (C) with a guanine (G).

In some embodiments, the primer has a mismatched nucleotide at the second nucleotide 3' downstream of the 3' end of the microsatellite, with respect to the primer. The mismatch is a substitution to any nucleotide which is mismatched to the corresponding nucleotide in the target sequence and the wild type sequence, and, in some embodiments, the mismatched nucleotide is G, A or T, preferably G. In some embodiments, the primer has a mismatched nucleotide at the second nucleotide 3' downstream of the 3' end of the microsatellite and at the first nucleotide 5' upstream of the 3' end of the microsatellite, with respect to the primer. In these embodiments, the second nucleotide 3' downstream of the 3' end of the microsatellite may be G, A or T, preferably G, and the first nucleotide 5' upstream of the 3' end of the microsatellite may be G, C or A,

preferably C or A. In some embodiments, the primer has a mismatched nucleotide at the second nucleotide 3' downstream of the 3' end of the microsatellite, at the first nucleotide 5' upstream of the 3' end of the microsatellite and at the second nucleotide 5' upstream of the 3' end of the microsatellite, with respect to the microsatellite. In these embodiments, the second nucleotide 3' downstream of the 3' end of the microsatellite may be G, A or T, preferably G, the first nucleotide 5' upstream of the 3' end of the microsatellite may be G, C or A, preferably C and the second nucleotide 5' upstream of the 3' end of the microsatellite may be G, C or A, preferably A. In some embodiments, the primer has a mismatched nucleotide at the second nucleotide 3' downstream of the 3' end of the microsatellite and at the second nucleotide 5' upstream of the 3' end of the microsatellite, with respect to the primer. In these embodiments, the second nucleotide 3' downstream of the 3' end of the microsatellite may be G, A or T, preferably G, and the second nucleotide 5' upstream of the 3' end of the microsatellite may be G, C or A, preferably A. In some embodiments, the primer has a mismatched nucleotide at the second nucleotide 3' downstream of the 3' end of the microsatellite, at the second nucleotide 5' upstream of the 3' end of the microsatellite and at fourth nucleotide 3' downstream of the 3' end of the microsatellite, with respect to the primer. In these embodiments, the second nucleotide 3' downstream of the 3' end of the microsatellite may be G, A or T, preferably G, the second nucleotide 5' upstream of the 3' end of the microsatellite may be G, C or A, preferably A, and the fourth nucleotide 3' downstream of the 3' end of the microsatellite may be A, G or C, preferably C.

In some embodiments, the primer has only one mismatch, which is at the second nucleotide 3' downstream of the 3' end of the microsatellite, with respect to the primer, and preferably the second nucleotide 3' downstream of the 3' end of the microsatellite is G. In some embodiments, the primer comprises or consists of the sequence of SEQ ID NO: 39, wherein X is A, G or T. In some embodiments, the primer comprises or consists of the sequence of SEQ ID NO: 3.

In some embodiments, the primer has mismatched nucleotides at the first, third and fourth nucleotides 5' upstream of the 3' end of the microsatellite, with respect to the primer. The mismatch is a substitution to any nucleotide which mismatches the corresponding nucleotide in the target sequence and the wild type sequence. In some embodiments, the primer has only three mismatched nucleotides, which are at the first, third and fourth nucleotides 5' upstream of the 3' end of the microsatellite, with respect to the primer. In these embodiments, it is preferred that the first nucleotide 5' upstream of the 3' end of the microsatellite is C, the third nucleotide 5' upstream of the 3' end of the microsatellite is A, and the fourth nucleotide 5' upstream of the 3' end of the microsatellite is A. In some embodiments, the primer comprises or consists of the sequence of SEQ ID NO: 40, wherein each of X₁, X₂ and X₃, independently, is A, C or G. In some embodiments, the primer comprises or consists of the sequence of SEQ ID NO: 15.

In some embodiments, the primer has mismatched nucleotides at the second nucleotide 5' upstream of the 3' end of the microsatellite, with respect to the primer, and at the fifth and twelfth nucleotides 3' downstream of the 3' end of the microsatellite, with respect to the primer. In some
5 embodiments, the primer has only three mismatched nucleotides, which are at the second nucleotide 5' upstream of the 3' end of the microsatellite and the fifth and twelfth nucleotides 3' downstream of the 3' end of the microsatellite. In these embodiments, it is preferred that the second nucleotide 5' upstream of the 3' end of the microsatellite is C, that the fifth nucleotide 3' downstream of the 3' end of the microsatellite is G, and that the twelfth nucleotide 3' downstream
10 of the 3' end of the microsatellite is C. In some embodiments, the primer comprises or consists of the sequence of SEQ ID NO: 41, wherein X_4 is A, C or G, X_5 is A, C or G and X_6 is C, G or T. In some embodiments, the primer comprises or consists of the sequence of SEQ ID NO: 31.

As explained above, the primer includes one, two, three or four nucleotides which are mismatched
15 to the target sequence containing the mutation in the microsatellite and which are also mismatched to a corresponding sequence containing the wild type microsatellite. This means that the primer includes at least one, but no more than four, nucleotides which are mismatched to the target sequence containing the mutation in the microsatellite and which are also mismatched to a corresponding sequence containing the wild type microsatellite.

The successful use of such primers in the context of a frameshift mutation in a microsatellite as disclosed herein came as a surprise to the present inventors. Before this invention, it was thought that it would not be possible to use a primer that anneals across the microsatellite in the target sequence to distinguish a frameshift mutation in said microsatellite due to the highly repeated
20 nature of these regions. It was thought that such primers could not be used to distinguish between the frameshift mutated and the wild type microsatellite sequence, since a primer designed to anneal to the frameshifted microsatellite would be thought also to anneal to the wild type sequence, even if the binding affinity is lower.

This primer approach allows for a distinction between the frameshift mutant and wild type microsatellite by using primers that are complementary to a target sequence containing a microsatellite having a frameshift mutation, except that the primer has at least one mismatch (and up to four mismatches) to this sequence, as well as being mismatched to a corresponding sequence containing the wild type microsatellite in order that this primer has an additional
25 mismatch to the wild type target sequence and, thus, further reduced affinity to the wild type target sequence. In other words, the primers are modelled on the sequence containing the microsatellite
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having a frameshift mutation, such that they comprise mismatches to the corresponding sequence having the wild-type microsatellite. The additional mismatch(es) between the primer and the microsatellite having a frameshift mutation results in further destabilisation and repulsion between the primer and the sequence containing the wild-type microsatellite.

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This distinction between a frameshifted microsatellite and a wild type microsatellite offers a very useful detection tool, where detecting such a frameshift is important, such as in cancers where such frameshifts may be a disease driver and/or a possible therapeutic target. As is disclosed herein, there are many microsatellites in which disease-relevant frameshift mutations can occur. For example, the frameshift may be in a microsatellite in any gene selected from ASTE1, ACVR22, TAF1B, KIAA2018, SLC22A9 and TGFBR2. Preferably, the frameshift is in a microsatellite in ASTE1 or TGFBR2.

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In another aspect of the invention, there is provided a kit for detection of a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule, wherein the mutation is a frameshift of the microsatellite as compared with the corresponding wild type microsatellite sequence, and wherein the kit comprises a first primer of the invention, and a second primer, wherein the second primer is configured to anneal to the target sequence 3' downstream of the microsatellite on the opposite strand of the DNA molecule to the strand on which the first primer is configured to anneal.

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In other words, this provides a primer pair for the detection method of the invention.

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In some embodiments, the frameshift mutation is in a microsatellite in the TGFBR2 gene and comprises the sequence according to residues 270 to 278 of SEQ ID NO: 20, wherein the first primer comprises the sequence defined by any one of SEQ ID NOs: 3, 4, 5, 7 or 39 and/or the second primer comprises the sequence defined by SEQ ID NO: 2. It will be understood that the use of the term "comprises" also includes primers that consist of the sequences listed above. The data disclosed herein demonstrates that these primers are particularly useful within the scope of the invention for use in the detection of frameshift mutations in a microsatellite within TGFBR2 (also referred to as a9, where a10 is the wild type microsatellite). However, alternatively, the frameshift mutation may be in a microsatellite in the ASTE1 gene, and the first primer can then comprise the sequence defined by any one of SEQ ID NOs: 11 to 16 and/or the second primer comprises the sequence defined by SEQ ID NO: 10. In some embodiments, the frameshift mutation is in a microsatellite in the ASTE1 gene and comprises the sequence according to residues 328 to 337 of SEQ ID NO: 33, wherein the first primer comprises the sequence defined

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by SEQ ID NO: 15, 31, 40 or 41, and/or the second primer comprises the sequence defined by SEQ ID NO: 10.

In some embodiments, the kit further comprises a third primer, wherein the third primer is configured to anneal 5' upstream of the region to which the second primer is configured to anneal and wherein the third primer is configured to anneal to the same strand as the first primer and the opposite strand to the second primer, or the kit comprises another control primer pair. For example, in the example wherein the frameshift mutation is in a microsatellite in the TGFBR2 gene and comprises the sequence according to residues 270 to 278 of SEQ ID NO: 19, then the third primer may comprise the sequence according to SEQ ID NO: 1. In the example wherein the frameshift mutation is in a microsatellite in the ASTE1 gene, then the third primer may comprise the sequence according to SEQ ID NO: 11 or SEQ ID NO: 38.

A control primer pair can be understood as being a positive control for any DNA amplification that is carried out with the kit, to ascertain that DNA amplification has been carried out successfully, so that e.g. no detection of a frameshift mutation with the primers of the invention can be confirmed as the absence of a frameshift in the sample, rather than an error or failure in the DNA amplification itself. A control primer pair can be any pair of primers that is able to amplify a target sequence that is known to be present in the sample (e.g. a conserved sequence between a frameshift mutation and a wild type sequence). Instead of a separate control primer pair, a third primer can be used with the second primer of the invention for the same purpose (i.e. a positive control).

Preferably, the sequence containing the wild type microsatellite comprises the sequence defined in SEQ ID NO: 19. As mentioned above, this is a microsatellite in TGFBR2 in which mutations can occur which can be linked with disease, such as gastric cancer (GC) and colorectal cancer (CRC). In some embodiments, the sequence containing the wild type microsatellite comprises the sequence defined in SEQ ID NO: 32. This is a microsatellite in ASTE1 in which mutations can occur which can be linked with disease, such as endometrial cancer and gastric cancer.

Preferably still, the third primer comprises the sequence defined by SEQ ID NO: 1. Again, it will be understood that the use of the term "comprises" also includes a primer that consist of this sequence. As described herein, this is a suitable example of a primer that can be used as one part of a positive control primer pair for detection of the a10 microsatellite in TGFBR2 and is demonstrated to function successfully as per the experimental data disclosed below. In some embodiments, the primer comprises the sequence defined by SEQ ID NO: 11. This is a suitable

example of a primer what can be used as part of a positive control primer pair for detection of the a11 microsatellite in ASTE1, and is demonstrated to function successfully in Figure 18.

In some embodiments, the target sequence comprising a microsatellite having a frameshift mutation comprises the sequence according to residues 270 to 278 of SEQ ID NO: 19 or SEQ ID NO: 20. As mentioned above, this is the frameshifted microsatellite in TGFBR2 that can be linked with disease, such as gastric cancer (GC) and colorectal cancer (CRC). In some embodiments, the target sequence comprising a microsatellite having a frameshift mutation comprises the sequence according to residues 328 to 337 of SEQ ID NO: 33. As mentioned above, this is the frameshifted microsatellite in ASTE1 that can be linked with disease, such as gastric cancer (GC) and endometrial cancer.

Preferably, the kit further comprises instructions for use.

In some embodiments, the kit further comprises the components for carrying out DNA amplification. Such components will be well known to the skilled person who is well acquainted with techniques for DNA amplification including polymerase chain reaction (PCR), loop mediated isothermal application (LAMP), nucleic acid sequence based amplification (NASBA or 3SR), strand displacement amplification (SDA), rolling circle amplification (RCA), and ligase chain reaction (LCR). The skilled person will know that such components can be provided separately, or can be bought as a commercial product as a “ready mix” of the necessary components required.

Optionally, these components may include a buffer, dNTPs and/or Taq polymerase. For example, this buffer may be provided as a 10X buffer and dNTPs may be provided at a 10mM concentration. In some embodiments, the buffer includes ammonium (NH_4^+) ions and, optionally, may be provided as 1X buffer. In some embodiments, the buffer is Key buffer, optionally 1X Key buffer.

In another aspect of the invention, there is provided a primer for DNA amplification comprising the sequence defined by any one of SEQ ID NO: 3, 4, 5, 7, 15, 31, 39, 40 or 41. Again, it will be understood that the use of the term “comprises” also includes primers that consist of the sequences listed above. As described above, the data disclosed herein demonstrates that these primers are particularly useful within the scope of the invention for use in the detection of frameshift mutations in a microsatellite within TGFBR2 (also referred to as a9, where a10 is the wild type microsatellite) and ASTE1 (also referred to as a10, where a11 is the wild type microsatellite). Alternatively, the primer may comprise the sequence defined by any of SEQ ID

NOs: 11 to 16, 31, 40 and 41. Again, it will be understood that the use of the term “comprises” also includes primers that consist of the sequences listed above

In a further aspect of the invention, there is provided a method for detecting a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule, wherein the mutation is a frameshift of the microsatellite as compared with the corresponding wild type microsatellite sequence using any of the above-detailed primers. The method comprises:

a) providing a first aliquot of a sample comprising human DNA,

b) adding to the first aliquot the necessary components for DNA amplification, a first primer and a second primer; wherein the first primer is suitable for detection of a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule and comprises a region of nucleotides that is complementary to the target sequence containing the microsatellite having a frameshift mutation except for between one and four, or between one and three, nucleotides which are mismatched to the target sequence containing the microsatellite having a frameshift mutation and which are also mismatched to a corresponding sequence containing the wild type microsatellite; and the second primer is configured to anneal to the target sequence 3' downstream of the microsatellite, to form a first reaction mix;

wherein the first primer is configured to anneal to the sense strand of the DNA molecule and the second primer is configured to anneal to the antisense strand of the DNA molecule, or the first primer is configured to anneal to the antisense strand of the DNA molecule and the second primer is configured to anneal to the sense strand of the DNA molecule,

c) carrying out DNA amplification on the first reaction mix,

d) detecting the presence of the frameshift mutation in the sample when an amplification product is produced from the DNA amplification of the first reaction mix. In some embodiments, the primer comprises between one and three nucleotides which are mismatched to the target sequence containing the microsatellite having a frameshift mutation and which are also mismatched to a corresponding sequence containing the wild type microsatellite.

As mentioned above, DNA amplification may include including polymerase chain reaction (PCR), loop mediated isothermal application (LAMP), nucleic acid sequence based amplification (NASBA or 3SR), strand displacement amplification (SDA), rolling circle amplification (RCA), and ligase chain reaction (LCR). However, preferably the DNA amplification used is PCR.

Preferably, DNA amplification is carried out in high stringency conditions. As mentioned above, high stringency conditions are conditions at which amplification is suboptimal so that the reaction only allows for successful DNA amplification where there is a very high level of matching to the

target sequence. In other words, the conditions are suboptimal for DNA amplification in order that primers with lower sequence matching to the target sequence are not able to anneal. Such suboptimal conditions can be generated by altering one or more of temperature, cycle number, ionic strength and the presence of certain organic solvents that allow pairing of nucleic acid sequences. In particular, this may include where the annealing step of PCR is carried out at a temperature that is at least 2%, 5%, or 10% higher than the recommended annealing temperature of the reaction. Alternatively, the annealing step of PCR is carried out at a temperature that is at least 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C or 10°C higher than the recommended annealing temperature of the reaction. In some embodiments, the annealing step of PCR is carried out at a temperature that is 4°C higher than the recommended annealing temperature of the reaction. In some embodiments, the annealing step is carried out at a temperature of 44°C, 44.5°C, 45°C, 45.5°C, 46°C, 46.5°C, 47°C, 47.5°C, 48°C, 48.5°C, 49°C, 49.5°C, 50°C, 50.5°C, 51°C, 51.5°C, 52°C, 52.5°C, 53°C, 53.5°C, 54°C, 54.5°C, 55°C, 55.5°C, 56°C, 56.5°C, 57°C, 57.5°C, 58°C, 58.5°C, 59°C, 59.5°C, 60°C, 60.5°C or 61°C. In some embodiments, the annealing step is carried out at a temperature between 54°C and 61°C, preferably between 56°C and 60°C, more preferably between 56°C and 59°C or between 56° and 58°C, and preferably wherein the frameshift is in a microsatellite in a TGFBR2 gene. In some embodiments, the annealing step is carried out at a temperature of 56.0°C, 56.1°C, 56.2°C, 56.3°C, 56.4°C, 56.5°C, 56.7°C, 56.8°C, 56.9°C, 57.0°C, 57.1°C, 57.2°C, 57.3°C, 57.4°C, 57.5°C, 57.6°C, 57.7°C, 57.8°C, 57.9°C or 58.0°C, and preferably the frameshift is in a microsatellite in a TGFBR2 gene. In some embodiments, the annealing step is carried out at a temperature of 57°C and preferably the frameshift is in a microsatellite in a TGFBR2 gene. In some embodiments, the annealing step is carried out at a temperature of between 44°C and 58°C, preferably between 45°C and 58°C or between 45°C and 56°C, more preferably between 45°C and 54.5°C, and preferably wherein the frameshift is in a microsatellite in a ASTE1 gene. In some embodiments, the annealing step is carried out at 48°C, 48.5°C, 49°C, 49.5°C, 50°C, 50.5°C, 51°C, 51.5°C, 52°C, 52.5°C, 53°C, 53.5°C, 54°C, 54.5°C, 55°C, 55.5 °C, 56°C, 56.5 °C, 57°C, 57.5°C or 58°C, preferably wherein the frameshift is in a microsatellite in a ASTE1 gene. In some embodiments, the annealing step is carried out at 44.5°C, 45°C, 45.5°C, 46°C, 46.5°C, 47°C, 47.5°C, 48°C, 48.5°C, 49°C, 49.5°C, 50.0°C, 50.5°C, 51.0°C, 51.5°C, 52.0°C, 52.5°C, 53.0°C, 53.5°C, 54.0°C, 54.5°C, 55.0°C, 55.5°C, 56.0°C, 56.5°C, 57.0°C or 57.5°C, preferably wherein the frameshift is in a microsatellite in a ASTE1 gene.

In some embodiments, the annealing step is carried out at a temperature of 56.0°C, 56.1°C, 56.2°C, 56.3°C, 56.4°C, 56.5°C, 56.7°C, 56.8°C, 56.9°C, 57.0°C, 57.1°C, 57.2°C, 57.3°C, 57.4°C, 57.5°C, 57.6°C, 57.7°C, 57.8°C, 57.9°C or 58.0°C, and the first primer comprises the sequence

defined by SEQ ID NO: 3, 4, 5, 7 or 39, preferably SEQ ID NO: 3 or 39. In some of these embodiments, the annealing step is carried out at a temperature of 57°C.

In some embodiments, the annealing step is carried out at a temperature of 48°C, 48.5°C, 49°C, 49.5°C, 50°C, 50.5°C, 51°C, 51.5°C, 52°C, 52.5°C, 53°C, 53.5°C, 54°C, 54.5°C, 55°C, 55.5 °C or 56°C, and the first primer comprises the sequence defined by SEQ ID NO: 15 or 40. Preferably, the annealing step is carried out at a temperature of 50°C, 50.5°C, 51°C, 51.5°C, 52°C, 52.5°C or 53°C.

In some embodiments, the annealing step is carried out at a temperature of 44.5°C, 45°C, 45.5°C, 46°C, 46.5°C, 47°C, 47.5°C, 48°C, 48.5°C, 49°C, 49.5°C, 50.0°C, 50.5°C, 51.0°C, 51.5°C, 52.0°C, 52.5°C, 53.0°C, 53.5°C, 54.0°C, 54.5°C, 55.0°C, 55.5°C, 56.0°C, 56.5°C, 57.0°C, 57.2°C or 57.5°C, and the first primer comprises the sequence defined by SEQ ID NO: 31 or 41. Preferably, the annealing step is carried out at a temperature of 45°C, 45.5°C, 46°C, 46.5°C, 47°C, 47.5°C or 48°C.

Additionally or alternatively, the high stringency conditions may include using a lower buffer concentration than is recommended for the reaction, such as 10%, 20%, 30%, 40%, 50%, 60% or 75% of the recommended concentration of a buffer in a reaction. In some embodiments, the buffer concentration which is lower than the recommended buffer concentration may be less than 0.1X, 0.2X, 0.3X, 0.4X, 0.5X, 0.6X or 0.75X. In some embodiments, the final concentration of buffer in the reaction mix(es) is between 0.1X and 2X, between 0.1X and 1.5X, between 0.1X and 1X, between 0.2X and 1X, between 0.3X and 1X, between 0.4X and 1X or between 0.5X and 1X. In some embodiments, the final concentration of buffer in the reaction mix(es) is 0.5X, 0.6X, 0.7X, 0.8X, 0.9X, 1X, 1.5X, or 2X.

Additionally or alternatively, the high stringency conditions may include using a more stringent buffer in the reaction, such as a buffer including ammonium ions (NH_4^+). In some embodiments, a buffer including ammonium (NH_4^+) ions is used at a standard concentration, such as 1X. In some embodiments, a buffer including ammonium (NH_4^+) ions is used (i.e. is in the reaction mix(es)) at a final concentration of between 1X and 2X, preferably 1X. In some embodiments, the buffer including ammonium (NH_4^+) ions is Key buffer. In some embodiments, the buffer is 1X Key buffer.

In some embodiments, the buffer includes potassium ions (K^+). In some embodiments, the buffer including potassium (K^+) ions is used at a final concentration of between 0.5X and 1X, preferably at 0.5X or 1X. In some embodiments, the buffer is standard Taq buffer (KCl).

5 In some embodiments, the final concentration of each of the forward and reverse primers in the reaction mix(es) is, independently, between 0.1 μ M and 1 μ M, between 0.1 μ M and 0.5 μ M, between 0.1 μ M and 0.4 μ M, between 0.1 μ M and 0.4 μ M or between 0.1 μ M and 0.3 μ M. In some embodiments, the final concentration of each of the forward and reverse primers in the reaction mix(es) is, independently, 0.1 μ M, 0.2 μ M or 0.3 μ M, preferably 0.2 μ M.

10 In some embodiments, the final concentration of the dNTPs in the reaction mix(es) is between 50 μ M and 500 μ M, between 50 μ M and 400 μ M, between 50 μ M and 300 μ M, between 100 μ M and 300 μ M, between 100 μ M and 200 μ M, between 150 μ M and 300 μ M, or between 150 μ M and 250 μ M. In some embodiments, the final concentration of the dNTPs in the reaction mix(es) is
15 100 μ M, 150 μ M, 200 μ M, 250 μ M or 300 μ M, preferably 200 μ M.

The high stringency conditions may additionally or alternatively include reducing the number of cycles in a PCR, such as carrying out 25 cycles, 20 cycles, 15 cycles or even 10 cycles, compared to 30, 35 or 40 cycles. In some embodiments, 30 or fewer, 25 or fewer, 20 or fewer, 15 or fewer
20 or 10 or fewer cycles of PCR are carried out. In some embodiments, more than one of these high stringency conditions is used. Preferably, 30 or 25 cycles of PCR are carried out.

In some embodiments, a recommended annealing temperature is provided by the manufacturer of a commercially obtained primer. The skilled person is aware of many publicly available tools
25 for calculating a recommended annealing temperature for a given primer. The skilled person is also aware that a recommended annealing temperature can be calculating by subtracting 3, 4, 5, or 6°C from the T_m (melting temperature) given for a particular primer. In one embodiment, the recommended annealing temperature for a primer is 4°C below the T_m of the primer under the conditions of the reaction. For example the T_m calculators are provided by New England BioLabs®
30 (found at <http://tmcalculator.neb.com/#!/main>) or Thermo Fisher Scientific® (found at <https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html>). The T_m calculator provided by Thermo Fischer Scientific® will also be known as the modified Allawi and SantaLucia method, as per Allawi and SantaLucia, 1997.

A recommended buffer concentration for a reaction mix is 1X. For example, in some embodiments 5µl of a 10X buffer is added to a reaction mix to a final volume of 50µl. In some embodiments, the buffer includes ammonium (NH₄⁺) ions. In some embodiments, the buffer is Key buffer.

- 5 In some embodiments, the annealing temperature is at least 57°C and the buffer includes potassium (K⁺) ions. Preferably the buffer is standard Taq (KCl) buffer. In these embodiments, the frameshift mutation in a microsatellite is preferably in a TGFBR2 gene (i.e. the target sequence is TGFBR2 having a frameshift mutation in a microsatellite).
- 10 A sample comprising human DNA may be any sample obtainable from a patient, for example, the sample may be a bodily fluid, a tissue, or cells. The sample may also be a liquid biopsy, such as plasma, which contains cell free DNA.

In some embodiments, the method further comprises:

- 15 a) also adding to the first aliquot at step b) of the method either:
- I) the second primer and a third primer, wherein the third primer is configured to anneal 5' upstream of the region to which the second primer is configured to anneal, and wherein the third primer is configured to anneal to the same strand as the first primer and the opposite strand to the second primer or
- 20 II) a control primer pair, to form the first reaction mix,
- and detecting that DNA amplification has been carried out successfully when an amplification product is produced from the DNA amplification using the second and third primers or the control primer pair; or
- b) providing a second aliquot of the sample comprising human DNA, adding to the second
- 25 aliquot the necessary components for DNA amplification and either:
- I) the first primer and a third primer, wherein the third primer wherein the third primer is configured to anneal 5' upstream of the region to which the second primer is configured to anneal, and wherein the third primer is configured to anneal to the same strand as the first primer and the opposite strand to the second primer or
- 30 II) a control primer pair, and
- carrying out DNA amplification on the second reaction mix, and detecting that DNA amplification has been carried out successfully when an amplification product is produced from the DNA amplification of the second reaction mix.
- 35 In some embodiments, the first primer is the primer of the invention described above and, therefore, has any of the features described above. For example, in some embodiments, the first

primer is configured to anneal across the length of the microsatellite having a frameshift mutation, and to at least two nucleotides flanking the 5' and/or at least two nucleotides flanking the 3' end of the microsatellite having a frameshift mutation. The first primer may anneal to at least three, four, or five nucleotides flanking one or both ends of the microsatellite having a frameshift mutation. In some embodiments, the first primer does not anneal across the length of the corresponding wild-type microsatellite sequence, and the 3' end of the first primer does not anneal to the corresponding sequence containing the wild-type microsatellite. This provides the advantage that the first primer anneals to the sequence containing the microsatellite having a frameshift mutation, but does not anneal to the corresponding sequence having the wild-type microsatellite. In some embodiments, the first primer anneals to one, two or three, preferably two or three, nucleotides flanking the 3' end of the microsatellite having a frameshift mutation (i.e. the first primer comprises two or three nucleotides 3' of the microsatellite, with respect to the first primer, which anneal to the corresponding sequence comprising the microsatellite having a frameshift mutation). In some embodiments, these two or three nucleotides form a GC-clamp. In some embodiments, the first primer anneals across the length of the microsatellite having the frameshift mutation and, 3' of the microsatellite having the frameshift mutation, with respect to the first primer, the first primer consists of two or three nucleotides which anneal to the nucleotides flanking the microsatellite having the frameshift mutation. This provides the advantage that the first primer annealed to the sequence containing the microsatellite having a frameshift mutation can be extended in the 5'-to-3' direction, thereby allowing replication of the sequence containing the microsatellite having the frameshift mutation. The nucleotides flanking the 3' end of the microsatellite having a frameshift mutation are described above.

In some embodiments, the first primer anneals to at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 nucleotides flanking the 5' end of the microsatellite having a frameshift mutation, with respect to the first primer. In some embodiments, at least 30%, 35%, 40%, 45%, 50%, 55%, 60% or 65% of the nucleotides of the first primer anneal to nucleotides flanking the 5' end of the microsatellite having a frameshift mutation, with respect to the first primer. In some embodiments, the part of the first primer which anneals to the nucleotides flanking the 5' end the microsatellite having a frameshift mutation, with respect to the first primer, also anneals to the corresponding sequence containing the wild-type microsatellite. This provides the advantage that the first primer is specific for the desired target sequence. The nucleotides flanking the 5' end of the microsatellite having the frameshift mutation are further described above.

In some embodiments, each of the primers consists of between 16 and 30 nucleotides. Preferably, the primer consists of between 16 and 25 nucleotides. Preferably still, the primer consists of

between 17 and 24 or between 17 and 23 nucleotides. That is to say, each of the primers independently consists of between 16 and 30 nucleotides. Preferably, each of the primers independently consists of between 1 and 25 nucleotides. Preferably still, each of the primers independently consists of between 17 and 24 nucleotides. Other preferred lengths of the primers are described above.

In some embodiments, at least one mismatched nucleotide is located in a position of the first primer that anneals 3' downstream of the microsatellite or within the microsatellite. Preferably, all of the mismatched nucleotides are located in a position of the first primer that anneals 3' downstream of the microsatellite or within the microsatellite. In some embodiments, all of the mismatched nucleotides are located in a position of the first primer that anneals within the microsatellite. Other preferred locations of at least one mismatch are described above.

In some embodiments, at least one mismatched nucleotide in the first primer is within three nucleotides 5' upstream or 3' downstream of the 3' end of the microsatellite. Preferably, all of the mismatched nucleotides in the first primer are within three nucleotides 5' upstream or 3' downstream of the 3' end of the microsatellite.

In some embodiments, at least one mismatched nucleotide in the first primer is a substitution of a thymine (T) with an adenine (A), a substitution of an adenine (A) with a guanine (G), a substitution of a thymine (T) with a cytosine (C), or a substitution of a cytosine (C) with a guanine (G).

In some embodiments, the DNA amplification is polymerase chain reaction (PCR), wherein the PCR comprises a plurality of cycles of denaturation, annealing and extension.

In some embodiments, the reaction mix(s) comprises 1x buffer (or 0.5x buffer), 0.4mM dNTPs, 0.2μM forward primer, and 0.2μM reverse primer. In some embodiments, the buffer includes ammonium (NH₄⁺) ions, and preferably is Key buffer.

In some embodiments, the method further comprises step d) further comprises running the product of the PCR reaction on a gel and visualising a band to confirm that DNA amplification has been successful.

This approach allows for a visual confirmation as to whether a PCR reaction has successfully generated an amplicon (i.e. amplified a target sequence from the sample). The skilled person is

well aware of how to run such a gel, and this will typically involve mixing the PCR reaction with a dye, loading this onto an agarose based gel, carrying out electrophoresis on the gel so as the dyed PCR reaction migrates towards the positive end of the gel forming a band that can be visualised. This provides an advantage over the conventional sequencing approach which can
5 take several days to provide a result, in that this PCR method can be carried out in several hours, or even less than an hour. As such, this method has the advantage that a clinically relevant finding (i.e. presence of a relevant mutation in a given disease) can be provided to a clinician or patient at a greater speed.

10 In a further embodiment, the method further comprises step f) cutting out the band for DNA sequencing. The advantage of running the PCR reaction on a gel as described above is that a band can be cut out of the gel and processed according to techniques known to the skilled person so that this can be sequenced. Sequencing is not essential, however this can allow for a further confirmation that the frameshift mutation is indeed present in the sample.

15 As is disclosed herein, there are many microsatellites in which disease-relevant frameshift mutations can occur. For example, the frameshift may be in a microsatellite in any gene selected from ASTE1, ACVR22, TAF1B, KIAA2018, SLC22A9 and TGFB2. In some embodiments, the frameshift mutation is in a microsatellite in TGFB2 or ASTE1.

20 In some embodiments, the frameshift mutation is in a microsatellite in the TGFB2 gene and the target sequence comprises the sequence according to residues 270 to 278 of SEQ ID NO: 19 or SEQ ID NO: 20, and the first primer comprises a sequence defined by any one of SEQ ID NOs: 3, 4, 5, 7 or 39 and/or the second primer comprises the sequence defined by SEQ ID NO: 2. In
25 some embodiments, the frameshift mutation is in a microsatellite in the ASTE1 gene and the target sequence comprises the sequence according to residues 328 to 337 of SEQ ID NO: 33, and the first primer comprises a sequence defined by SEQ ID NO: 15, 33, 40 or 41 and/or the second primer comprises the sequence defined by SEQ ID NO: 10.

30 In some embodiments, the microsatellite is in a TGFB2 gene, the sequence comprising the wild type microsatellite comprises the sequence according to SEQ ID NO: 19, and the third primer comprises the sequence defined by SEQ ID NO: 1. In some embodiments, the microsatellite is in a ASTE1 gene, the sequence comprising the wild type microsatellite comprises the sequence according to SEQ ID NO: 32, and the third primer comprises the sequence defined by SEQ ID
35 NO: 11 or SEQ ID NO: 38. As described above, the data disclosed herein demonstrates that these primers are particularly useful within the scope of the invention for use in the detection of

frameshift mutations in a microsatellite within TGFBR2 (also referred to as a9, where a10 is the wild type microsatellite) or ASTE1.

In some embodiments, the sample is a liquid biopsy comprising cell free DNA, preferably wherein the liquid biopsy is plasma or serum. Alternatively, the sample is a tissue biopsy. Preferably, the sample is obtained from a human. Detection from liquid biopsies is particularly advantageous, as such biopsies are easily obtained from patients and are often easier to extract DNA from than e.g. a tumour tissue biopsy which may be necrotic and/or have variable DNA content making analysis more difficult. As previously mentioned, tumours are often heterogeneous and thus a tumour biopsy may not be representative of the whole tumour, and instead only the part that is sampled. Liquid biopsy overcomes this issue by providing a representative sample.

In some embodiments, the method further comprises determining that a patient suffering from a disease or disorder associated with a frameshift mutation is suitable for a treatment targeting said frameshift mutation if the frameshift mutation is detected in step d) in a sample from the patient. Preferably, the disease or disorder associated with a frameshift mutation is a cancer.

In some embodiments, the disease or disorder is colorectal cancer (CRC), gastric cancer (GC) or Lynch Syndrome, and the treatment targeting the frameshift mutation is FMPV-1, wherein the frameshift mutation is in a microsatellite in the TGFBR2 gene and comprises the sequence according to residues 270 to 278 of SEQ ID NO: 19 or SEQ ID NO: 20. In some embodiments, the disease or disorder is endometrial cancer or gastric cancer and the treatment targeting the frameshift mutation is FMPV-2, wherein the frameshift mutation is in a microsatellite in the ASTE1 gene and comprises the sequence according to residues 328 to 337 of SEQ ID NO: 33.

Preferably, the method further comprises step e) of treating the patient with FMPV-1 or FMPV-2. FMPV-1 is a peptide vaccine as described in WO 2020/239937A1, which is incorporated herein by reference. FPMV-1 is also known as fsp2. FMPV-2 is a peptide vaccine as described in WO2021/239980, which is incorporated herein by reference. FMPV-2 is also known as fsp8.

Optionally, a third primer, or another control primer pair, can also be used for DNA amplification from the sample in order to provide a positive control for the detection methods of the invention. In some embodiments, the microsatellite is in a TGFBR2 gene, the sequence comprising the wild type microsatellite comprises or consists of the sequence according to SEQ ID NO: 19 or SEQ ID NO: 34, and wherein the third primer comprises the sequence defined by SEQ ID NO: 1. In some embodiments, the microsatellite is in a ASTE1 gene, the sequence comprising the wild type

microsatellite comprises or consists of the sequence according to SEQ ID NO: 32 or SEQ ID NO: 36, and the third primer comprises the sequence defined by SEQ ID NO: 11 or SEQ ID NO: 38.

In some embodiments, the first primer comprises the sequence defined by any one of SEQ ID NO: 3, 4, 5, 7, 15, 31, 39, 40 or 41, and/or the second primer comprises the sequence defined by SEQ ID NO: 2 or 10.

In another aspect of the invention, there is provided a method of diagnosing a disease associated with a frameshift mutation in a microsatellite, comprising carrying out any of the methods of the invention. As described above, frameshift mutations in microsatellites have been linked with a number of diseases, including but not limited to: Lynch Syndrome, cystic fibrosis, Crohn's disease, and cancer including colon, gastric, endometrium, ovarian, hepatobiliary tract, urinary tract, brain and skin cancers. In particular cancer-associated frameshift mutations in microsatellites may be detected in any number of genes including but not limited to: ASTE1, ACVR22, TAF1B, KIAA2018, SLC22A9 and TGFBR2.

In any of the above methods, it is preferable that the sample is a liquid biopsy comprising cell free DNA, preferably wherein the liquid biopsy is plasma.

In any of the above methods, it is preferable that the DNA amplification is PCR, and that the PCR is carried out in high stringency conditions, optionally wherein the high stringency conditions comprise at least one of:

a) carrying out the annealing step of PCR at a temperature that is at least 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C or 10°C higher than the recommended annealing temperature of the reaction, preferably at a temperature between 53°C and 60°C;

b) carrying out the annealing step of PCR for only 30 seconds, preferably 15 seconds, per cycle;

c) carrying out the DNA amplification in a buffer concentration that is less than 0.1X, 0.2X, 0.3X, 0.4X, 0.5X, 0.6X or 0.75X ;

d) carrying out the DNA amplification in a buffer comprising ammonium ions;

e) reducing the number of cycles of PCR to 25, 20, 15 or 10 cycles, optionally 30 or fewer, 25 or fewer, 20 or fewer, 15 or fewer, or 10 or fewer cycles. The high stringency conditions may be any of those described above.

For example, in some embodiments, the high stringency conditions comprise carrying out the annealing step at a temperature that is at least 4°C higher than the recommended annealing

temperature, and/or carrying out the DNA amplification in a buffer comprising ammonium (NH_4^+) ions, and/or carrying out the annealing step of PCR for 15 seconds per cycle, and/or reducing the number of cycles of PCR to 25 or fewer. In some embodiments, the high stringency conditions comprise carrying out the annealing step at a temperature that is at least 4°C higher than the recommended annealing temperature, preferably at a temperature which is 4°C higher than the recommended annealing temperature, carrying out the DNA amplification in buffer comprising ammonium (NH_4^+) ions at 1X concentration, preferably wherein the buffer is Key buffer, carrying out the annealing step of PCR for 15 seconds, and reducing the number of cycles of PCR to 25.

In some embodiments, the high stringency conditions comprise carrying out the annealing step at a temperature of 58°C in a buffer including potassium (K^+) ions, preferably standard Taq (KCl) buffer. In these embodiments, it is preferable that the frameshift mutation in the microsatellite is in TGFBR2.

The invention will now be demonstrated in the below non-limiting experimental examples.

EXAMPLE I – Designing primers for detection of frameshifted mutated TGFBR2

Primer design typically follows particular rules to ensure a desirable yield of a single, specific amplicon (fragment). Most of the time these rules are easily taken in accordance - even when distinguishing between highly similar sequences. However, since the only difference, in the case of distinguishing a frameshift in a microsatellite, is the length of the microsatellite (one nucleotide difference) this is not applicable. The flanking regions of the satellite have the exact same sequence in both variants of DNA, making the affinity of a primer between them close to equal. In turn this is singlehandedly the largest obstacle to overcome in defining the detection test, since the primer design is locked to this exact sequence/area - without a possibility to change the parameters too much. The strategy used in designing mutant detection primers is therefore to create mismatches with the wild type sequence, and in that way induce enough repulsive forces and block primer annealing to the wild type DNA.

In a series of 14 experiments a panel of PCR primers (Table 1) for detection of frameshift mutated TGFBR2 was designed and tested. Initially a first set of primers were designed and tested for amplification of TGFBR2 DNA. Based on the results from the preceding experiment(s), the primers were optimized and re-tested. Synthetic TGFBR2 DNA fragments comprising the wild type and the mutant microsatellites, a10 and a9, respectively, were used for the PCR set up (SEQ ID NOs: 19 and 20 respectively).

Table 1

Primer	SEQ	5' - 3' Direction	Sequence
	ID NO:		
P1	1	Reverse	CAGTGT CATAGAGCACATTCCT
P2	2	Forward	AGACAGTTTGCCATGACCC
P4	3	Reverse	GTCTCACCAGGCTTTTTTTTTTC <u>G</u>
P5	8	Reverse	GTCTCACCAGGCTTTTTTTTTTCC
P6	4	Reverse	GTCTCACCAGGCTTTTTTTTTT <u>ACG</u>
P10	5	Reverse	CACCAGGCTTTTTTTTTT <u>A</u> T <u>C</u> GTTTC

EXAMPLE II – Testing functionality of primers

The first two primers (P1 & P2) act as the positive control regarding the detection test, to ensure reliable results and that all criteria to run the PCR are met (no signal from the control = unsuccessful PCR). In addition, the positive control ensures that the correct length and sequence of cfDNA (circulating free DNA) that is needed to perform the detection / determination of TGFbR2 variant is present. A P1 – P2 derived amplicon (249bp) encompasses all the other designed primer targets (corresponding sequences) and is not mutant specific.

The mutant primers (P4, P5, P6 and P10) were designed with mutant variant of TGFbR2 as primary target and harbours the 9A microsatellite sequence. Just by having a shorter sequence of 9A instead of 10A, there are mismatches between the wild type and the 3'-end of the primers. By introducing a single nucleotide substitution (randomly selected) on this end of primers P4, P6 and P10, the mismatch repulsion can be reinforced. Amplicons (102bp – 169bp) are produced in conjugation with one of the control primers.

In order to determine the required amount of template DNA for PCR reactions, PCR test runs were conducted under standard PCR conditions, using protocols and calculated temperatures from NEB (New England BioLabs), using different amounts of template DNA. The results of these test runs can be seen in the electrophoresis gels shown in Figure 1.

In particular, it was found that even a very small amount of template (less than 2pg, as can be seen in Figure 1B) achieves a good yield and high resolution bands in the gel. Furthermore, the amplicons have the correct expected length showing high specificity

It was found that all primers were functional, with successful amplification from the 9a microsatellite (mutant) and 10a microsatellite (wild type) template DNA under standard PCR conditions (see Table 2), pairing each primer with P2 (or with P1 in the case of P2). The product of these PCR reactions were stained with DNA staining and run on a pre-made agarose-TAE mixture (2%) by performing electrophoresis at 100V for approximately 20 to 30 minutes until the running front reached the end of the gel. The result of this electrophoresis demonstrated successful amplification from both template DNA types with strong bands visible in all lanes. However, the primers did not differentiate between the microsatellite containing the frameshift mutation (9a) (mutant) and the microsatellite not containing the frameshift mutation (10a) (wild type).

EXAMPLE III – Testing P4 in suboptimal conditions (high stringency)

Therefore, this experimental approach was repeated using more stringent PCR conditions, using a slightly higher annealing temperature of 53°C, and fewer cycles of only 25 (see Table 2). The result of this condition change was that there was less amplification of the microsatellite not containing the frameshift mutation (10a) (wild type) which can clearly be seen in Figure 2 by reduced band sizes for primer P4 where the signal is clearly diminishing in the 10a template.

The experiment was then repeated using the P2 and P4 primers under even more stringent conditions than for the experiment shown in Figure 2, increasing the annealing temperature further to 55°C and shortening the annealing and extension steps by 15 seconds and 20 seconds respectively. These conditions are shown below in Table 2. These conditions provided even further improved distinction between the 10a template (wild type) and 9a template (mutant) with an extremely weak, almost invisible band for the wild type template, as can be seen in Figure 3. The same was not found with P5, where no distinction between the 10a template (wild type) and 9a template (mutant) was found using this primer even in stringent conditions (data not shown).

Table 2

<i>Standard stringency conditions</i>		
Step	Temp	Time
Initial denaturation	95°C	30 sec.
Denaturation (30 cycles)	95°C	30 sec.
Annealing (30 cycles)	52°C	45 sec.
Extension (30 cycles)	68°C	30 sec.
Final extension	68°C	5 min.
Hold	4°C	-
<i>High stringency conditions used in Figure 2</i>		
Step	Temp	Time
Initial denaturation	95°C	30 sec.

Denaturation (25 cycles)	95°C	30 sec.
Annealing (25 cycles)	53°C	45 sec.
Extension (25 cycles)	68°C	30 sec.
Final extension	68°C	5 min.
Hold	4°C	-
High stringency conditions used in Figure 3		
Step	Temp	Time
Initial denaturation	95°C	30 sec.
Denaturation (25 cycles)	95°C	30 sec.
Annealing (25 cycles)	55°C	35 sec.
Extension (25 cycles)	68°C	10 sec.
Final extension	68°C	5 min.
Hold	4°C	-

EXAMPLE IV – Testing P4, P6 and P10 in suboptimal conditions (higher stringency)

Again, in order to use the primers to distinguish between the mutant (9a) and wild type (10a) template DNA, more stringent PCR conditions were used as per Table 3, with a higher annealing temperature and a shorter annealing step used. In addition, the PCR reaction was carried out twice per primer pair, once at the standard buffer concentration, and once at a low buffer concentration (reduced to 50% of the standard concentration). Only primer pairs using P6 and P10 were examined in this particular experiment. As can be seen in Figure 4, where low buffer concentration samples are marked with an asterisk (*), this resulted in a clear strong band only in the sample with the low buffer concentration using the P10 primer in the mutant, and not in the wild type template.

Table 3

Standard stringency conditions		
Step	Temp	Time
Initial denaturation	95°C	30 sec.
Denaturation (25 cycles)	95°C	15 sec.
Annealing (25 cycles)	51°C	30 sec.
Extension (25 cycles)	68°C	10 sec.
Final extension	68°C	5 min.
Hold	4°C	-
High stringency conditions used in Figure 4		
Step	Temp	Time
Initial denaturation	95°C	30 sec.
Denaturation (25 cycles)	95°C	15 sec.
Annealing (25 cycles)	56°C	15 sec.
Extension (25 cycles)	68°C	45 sec.
Final extension	68°C	5 min.
Hold	4°C	-

In order to optimise the amplification by the designed primers from the mutant template, further experiments were carried out using P4, P6 and P10 (as well as P1 as a positive control) using the mutant template DNA (9a) at a number of different conditions.

- 5 In particular, in the experiments shown in Figures 5A-C used annealing temperatures of 55°C, 56°C, and 58°C respectively as can be seen below in Table 4. Again, a “low” buffer concentration (denoted by an asterisk, *) in the experiments shown in Figures 5A-6C comprised a buffer concentration of around 50% of the standard buffer concentration. As can be seen, stronger signals were seen in Figures 5B and 5C, particularly at the low buffer concentration. Of note, the
- 10 P6 primer showed amplification here, confirming the hypothesis that the lack of amplification in the previous experiments was due to human error.

Table 4

PCR conditions used in Figure 5A		
Step	Temp	Time
Initial denaturation	95°C	2 min.
Denaturation (30 cycles)	95°C	15 sec.
Annealing (30 cycles)	55°C	15 sec.
Extension (30 cycles)	68°C	45 sec.
Final extension	68°C	5 min.
Hold	4°C	-
PCR conditions used in Figure 5B		
Step	Temp	Time
Initial denaturation	95°C	2 min.
Denaturation (25 cycles)	95°C	15 sec.
Annealing (25 cycles)	56°C	15 sec.
Extension (25 cycles)	68°C	45 sec.
Final extension	68°C	5 min.
Hold	4°C	-
PCR conditions used in Figure 5C		
Step	Temp	Time
Initial denaturation	95°C	2 min.
Denaturation (25 cycles)	95°C	15 sec.
Annealing (25 cycles)	58°C	15 sec.
Extension (25 cycles)	68°C	45 sec.
Final extension	68°C	5 min.
Hold	4°C	-

- Further PCR reactions were carried out using primer pairs at an even higher annealing
- 15 temperature of 60°C (see Table 5 below) at both standard and low buffer concentrations (Figure 6). A strong signal was seen in the control (P1) at both the standard and low buffer concentrations.

This higher annealing temperature allowed for the selective amplification of mutant template by primer P6 in combination with primer P2, with no detectable amplification of wild type template, which can be seen in Figure 6A.

In addition, three primer reactions were also carried out in the same conditions, which can be seen in Figure 6B. Three primer reactions contained P1, P2 and either P4, P6 or P10, and it was expected that two bands would be seen at 249bp and 102bp. No signal was seen in the low buffer concentration reactions, and only a single band was seen in the standard buffer concentration reactions.

The same conditions were used again (as per Table 5) to perform further PCR reactions, but also including primer P4 (Figure 7). In these experiments, it was found that a strong band from the positive control (P1) was seen in the standard buffer concentration, and a weaker band was seen in the low buffer concentration. At the standard buffer concentration, amplification with P10 was seen in both the mutant and wild type templates, however in the low buffer concentration, it was seen that amplification with P10 was seen from the mutant template but not the wild type template (Figure 7). Thus, it was found that P10 in the low buffer concentration had no affinity towards the wild type sequence.

Again, three primer reactions were carried out in the same conditions, and in this experiment, whilst only one weak band was detected at the low buffer concentration, two bands (one strong and one weak) were seen in the standard buffer concentration.

Table 5

PCR conditions used in Figure 6A, 6B and 7		
Step	Temperature	Time
Initial denaturation	95°C	2 min.
Denaturation (25 cycles)	95°C	15 sec.
Annealing (25 cycles)	60°C	15 sec.
Extension (25 cycles)	68°C	45 sec.
Final extension	68°C	5 min.
Hold	4°C	-

EXAMPLE V – Testing functionality of primers P4.1, P4.2 and P10.1

As per the above example, further primers P4.1, P4.2 and P10.1, which were further modified from primers P4 and P10 were also tested by pairing with primer P2 and carrying out PCR (conditions are detailed below in Table 6) followed by gel electrophoresis (shown in Figure 8). This experiment showed that all three of these primers are functional as they showed amplification against both the mutant (9a) and wild type (10a) templates, as can be seen in Figure 8, which shows strong banding. A significantly reduced band can be seen for P4.1 in the mutant template relative to the equivalent band in the wild type template.

Table 6

PCR conditions used in Figure 8		
Step	Temperature	Time
Initial denaturation	95°C	2 min.
Denaturation (25 cycles)	95°C	15 sec.
Annealing (25 cycles)	52°C	15 sec.
Extension (25 cycles)	68°C	45 sec.
Final extension	68°C	5 min.
Hold	4°C	-

EXAMPLE VI – Testing candidate primers in higher stringency PCR conditions

To use the primers to distinguish between the mutant (9a) and wild type (10a) template, PCR reactions were conducted using two different types of buffer: standard Taq buffer which does not comprise ammonium ions, and Key buffer which comprises ammonium ions (NH_4^+).

Firstly, PCR reactions on mutant template using Taq buffer or Key buffer were used in the conditions shown in Table 7 and run on an electrophoresis gel as shown in Figure 9A. It was found that relatively uniform signal strength was seen regardless of which buffer was used, with the exception of primer P4.2 where the reaction in Key buffer showed minimal signal.

These reactions were then repeated with a slightly higher annealing temperature (again shown in Table 7), and run on an electrophoresis gel as shown in Figure 9B. This experiment showed comparable results as those seen in Figure 9A, however the signal produced by the reaction with primer P10.1 in Key buffer was significantly reduced. The signal produced by the reaction with primer P4.2 was more distinguishable in this experiment.

The immediate difference between signals produced by some PCR samples containing key buffer and Taq-buffer suggests that stabilization of primer/template interaction is highly affected. Having a higher number of mismatches between designed primers and wildtype DNA could lead to elimination of amplicons produced from these samples using key buffer. From the results it is apparent that several mutant template DNA and primer samples have equal signal strength regardless of buffer variant, and it is anticipated that the interaction remains in these samples and amplicons are produced whereas wild type template have decreased primer annealing. Control primers appear unaffected by the buffer variants and temperatures tested. Since the samples with P4.2 and key buffer were not consistent through the two experiments is indicative that the P4.2 stock were of poor condition.

Table 7

PCR conditions used in Figure 9A		
Step	Temperature	Time
Initial denaturation	95°C	2 min.
Denaturation (25 cycles)	95°C	15 sec.
Annealing (25 cycles)	56°C	15 sec.
Extension (25 cycles)	68°C	45 sec.
Final extension	68°C	5 min.
Hold	4°C	-
PCR conditions used in Figure 9B		
Step	Temperature	Time
Initial denaturation	95°C	2 min.
Denaturation (25 cycles)	95°C	15 sec.
Annealing (25 cycles)	58°C	15 sec.
Extension (25 cycles)	68°C	45 sec.
Final extension	68°C	5 min.
Hold	4°C	-

Further experiments were carried out in the same conditions as Figure 9A shown in Table 7, using primers P4, P4.1, P4.2 and P6 in combination with P2 on both mutant and wild type template DNA. These reactions were also run on electrophoresis gels as can be seen in Figure 10. Reactions in Figure 10A were carried out in standard Taq buffer, and reactions in Figure 10B were carried out in Key buffer, which contains ammonium ions (NH_4^+).

In samples containing standard Taq-buffer there were sufficient amplicon produce to give strong bands with the various primers and templates (Figure 10A). Samples containing the key buffer showed lack of primer annealing and thus product yield as visualized by weak/disappearing bands in some primer/template combinations (Figure 10B). With mutant template DNA, P6 had heavily reduced functionality. Turning to the wild type template DNA, there were loss of function in P4, P6, and fractioned/reduced performance in P4.2.

The experiments were then repeated at a slightly higher annealing temperature of 59°C, and run on electrophoresis gels, which can be seen in Figure 11, with reactions in standard Taq buffer shown in Figure 11A and reactions in Key buffer shown in Figure 11B. As seen in the previous experiment, all samples yielded strong signals on the gel regardless of template DNA. Looking at samples with mutated DNA as template and Key buffer, the only primer which did not produce amplicon (or produced a low amount) in this combination is P6. With wild type DNA template and key buffer, weak/disappearing bands were noticed in samples with P4.2 and P6.

Looking at the results from Figures 10 and 11, it is evident that key buffer (ammonium, NH_4^+) has a significant destabilizing effect that prevents the annealing of (selected) primers to template DNA. In addition, slight alterations in temperature plays a significant role in loss- or gain of function (in

annealing). Since the designed primers will inherently have more mismatches in combination with wild type sequence as template DNA in comparison to mutant sequence, the influence of key buffer is significantly higher. Primer/template annealing remain functional with mutant TGF β R2 sequence at a wider area of parameters.

5

EXAMPLE VII - Detection of TGF β R2 a10→a9 frameshift mutation in cell free DNA (cfDNA) of liquid biopsies from MSI-colorectal cancer patients

Methods

cfDNA was extracted from patient plasma samples using a cfDNA extraction kit (Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit, category number 55100, www.norgenbiotek.com). The patient samples were bought from Indivumed GmbH (www.indivumed.com) and were all taken at the same point in treatment of the individual patient – T0 (baseline). Patients had colorectal cancer (CRC), were MSI-H or MSS, and were aged 42-73.

15 i) Sequencing of cfDNA

Sequencing of cfDNA fragments present in the plasma samples was done by performing PCR with a high-fidelity polymerase (OneTaq DNA Polymerase, New England Biolabs, www.international.neb.com) and standard conditions, using 1X OneTaq-buffer (KCl). The high-fidelity polymerase had 3'-5' exonuclease activity and ensured that the sequence of the microsatellite, where polymerases are prone to "slipping" due to the high number of single-nucleotide repeats, was correctly synthesized. Synthetic TGF β R2 template DNA was used as controls (wild-type (same sequence as SEQ ID NO: 19) and 9a mutant variant (same sequence as SEQ ID NO: 20)), in order to verify that the sequencing data of the microsatellite is correct and that the read-out of the electrophoresis gel is consistent compared to known sequences. Primers used in PCR for sequencing purposes were P1 and P2.

The PCR conditions were as set out in Table 8.

Table 8

Thermocycling conditions (OneTaq)		
Step	Temperature	Time
Initial denaturation	94°C	2 min.
Denaturation (30 cycles)	94°C	15 sec.
Annealing (30 cycles)	52°C	15 sec.
Extension (30 cycles)	68°C	20 sec.
Final extension	68°C	5 min.
Hold	4°C	-

The PCR product was purified using gel electrophoresis. The band with fragments/amplicons with the correct length was cut from the gel and extracted using a gel-extraction kit (VWR peqGOLD Gel Extraction Kit, category number 13-2500-01, www.vwr.com). Extracted PCR product was then sent for sequencing, which was performed by Eurofins Genomics (www.eurofinsgenomics.eu).

ii) Detection of mutated TGF β R2 cfDNA

cfDNA from the patient plasma samples, and synthetic wild-type and a9 mutant TGF β R2 (SEQ ID NOS: 19 and 20) as controls, was amplified by PCR, using the conditions in Table 10. Primers P1 and P2 were used for positive controls, and primers P2 and P4 were used for detection of mutated TGF β R2, as shown in Table 9. In Table 9, the "Primer" column indicates only whether P1 or P4 was used, as primer P2 was used in all tubes. In the "Sample" column, "synth" indicates that synthetic DNA was used as the template (i.e. the control samples), rather than cfDNA obtained from the patient plasma samples. The buffer used was 1X Key buffer ((NH₄)₂SO₄). The PCR products were subjected to gel electrophoresis, and then sequenced.

Table 9

Tube	Primer	DNA template	Buffer	Sample
1	P1	MSI BD_001	Key	T0
2	P4	MSI BD_001	Key	T0
3	P1	MSI BD_002	Key	T0
4	P4	MSI BD_002	Key	T0
5	P1	MSI BD_003	Key	T0
6	P4	MSI BD_003	Key	T0
7	P1	MSI BD_004	Key	T0
8	P4	MSI BD_004	Key	T0

Tube	Primer	DNA template	Buffer	Sample
9	P1	MSI BD_005	Key	T0
10	P4	MSI BD_005	Key	T0
11	P1	MSS BD_006	Key	T0
12	P4	MSS BD_006	Key	T0
13	P1	MSS BD_007	Key	T0
14	P4	MSS BD_007	Key	T0
15	P1	MSS BD_008	Key	T0
16	P4	MSS BD_008	Key	T0

Tube	Primer	DNA template	Buffer	Sample
17	P1	MSS BD_009	Key	T0
18	P4	MSS BD_009	Key	T0
19	P1	MSS BD_010	Key	T0
20	P4	MSS BD_010	Key	T0
21	P1	F1, mutant	Key	Synth.
22	P1	F2, wildtype	Key	Synth.
23	P4	F1, mutant	Key	Synth.
24	P4	F2, wildtype	Key	Synth.

Table 10

Thermocycling conditions		
Step	Temperature	Time
Initial denaturation	95°C	30 sec.
Denaturation (25 cycles)	95°C	15 sec.
Annealing (25 cycles)	56°C	15 sec.
Extension (25 cycles)	68°C	45 sec.
Final extension	68°C	5 min.
Hold	4°C	-

Results

TGFbR2 DNA was present in cfDNA from all (10/10) CRC patients tested. PCR amplification using the primers, followed by gel electrophoresis, showed that a TGFbR2 a10→a9 frameshift was present in all (5/5) MSI-CRC patients and none (0/5) of the MSS-CRC patients tested (Figure 12). These results were confirmed by sequencing of the PCR products of correct size extracted from the electrophoresis gel. The sequenced PCR products contained the entire a9/a10 microsatellite, as relevant, and the sequencing data was shown to correspond to the PCR results.

PCR using the selected a9 modelled primer (P4) yielded a PCR product that was clearly detectable on the electrophoresis gel only for cfDNA from the MSI-CRC patients (5/5). The TGFbR2 cfDNA fragments were long enough to contain the microsatellite and the regions that the control primer pair anneals to (250bp).

The results show that TGFbR2 a10→a9 frameshift DNA is present in cfDNA of liquid biopsies from MSI-CRC patients, which establishes cell free TFGbR2 frameshift DNA as a potential biomarker for early detection of hereditary CRC (Lynch Syndrome) as well as for monitoring cancer progression and remission of sporadic MSI-CRC.

The sequences of the cfDNA from each patient, extracted from the electrophoresis gels, are shown in Table 11, with the length of the microsatellite shown in parentheses at the end of each sequence.

5 Table 11

Patient	SEQ ID NO:	Sequence
BD_001 (MSI-H)	21	TTGGCACAGATCTCAGGTCCCACACCCTTAAGAGAAGAAA ACTCACCTTCTGAGAAGATGATGTTGTCATTGCACTCATCA GAGCTACAGGAACACATGAAGAAAGTCTCACCAGGCTTTT TTTTTCCTTCATAATGCACTTTGGAAAAGCAGCATCTTCCA AAATAAAGTCATGGTAGGGGAGCTTGGGGTCATGGCAAA CTG (A9)
BD_002 (MSI-H)	22	TGGCACAGATCTCAGGTCCCACACCCTTAAGAGAAGAAA CTCACCTTCTGAGAAGATGATGTTGTCATTGCACTCATCA GAGCTACAGGAACACATGAAGAAAGTCTCACCAGGCTTTT TTTTTCCTTCATAATGCACTTTGGAGAACCAGCATCTTCCA AAATAAAGTCATGGAAGGGGAGCTTGGGGTCAGGGCAAA CTG (A9)
BD_003 (MSI-H)	23	CCGGAAAAAATTGGCACAGATCTCAGGTCCCACACCCTTA AGAGAAGAAAACCTCACCTTCTGAGAAGATGATGTTGTCAT TGCACTCATCAGAGCTACAGGAACACATGAAGAAAGTCTC ACCAGGCTTTTTTTTTTCCTTCATAATGCACTTTGGAGAAGC AGCATCTTCCAGAATAAAGTCATGGTAGGGGAGCTTGGG GTCATGGCAAACGTCTAA (A9)
BD_004 (MSI-H)	24	ACAGATCTCAGGTCCCACACCCTTAAGAGAAGAAAACCTCA CCTTCTGAGAAGATGATGTTGTCATTGCACTCATCAGAGC TACAGGAACACATGAAGAAAGTCTCACCAGGCTTTTTTTTT CCTTCATAATGCACTTTGGAAAAGCAGCATCTTCCAAAATA AAGTCATGGTAGGGGAGCTTGGGGTCATGGCA (A9)
BD_005 (MSI-H)	25	TGGCACAGATCTCAGGTCCCACACCCTTAAGAGAAGAAA CTCACCTTCTGAGAAGATGATGTTGTCATTGCACTCATCA GAGCTACAGGAACACATGAAGAAAGTCTCACCAGGCTTTT TTTTTCCTTCATAATGCACTTTGGAGAAGCA (A9)
BD_006 (MSS)	26	ATTGGCACAGATCTCAGGTCCCACACCCTTAAGAGAAGAA AACTCACCTTCTGAGAAGATGATGTTGTCATTGCACTCATC

		AGAGCTACAGGAACACATGAAGAAAGTCTCACCAGGCTTT TTTTTTCCTTCAA (A10)
BD_007 (MSS)	27	TGGCACAGATCTCAGGTCCCACACCCTTAAGAGAAGAAAA CTCACCTTCTGAGAAGATGATGTTGTCATTGCACTCATCA GAGCTACAGGAACACATGAAGAAAGTCTCACCAGGCTTTT TTTTTTCCTTCATAAG (A10)
BD_008 (MSS)	28	ACAGATCTCAGGTCCCACACCCTTAAGAGAAGAAAACTCA CCTTCTGAGAAGATGATGTTGTCATTGCACTCATCAGAGC TACAGGAACACATGAAGAAAGTCTCACCAGGCTTTTTTTTTT TCCTTCTTA (A10)
BD_009 (MSS)	29	TGGCACAGATCTCAGGTCCCACACCCTTAAGAGAAGAAAA CTCACCTTCTGAGAAGATGATGTTGTCATTGCACTCATCA GAGCTACAGGAACACATGAAGAAAGTCTCACCAGGCTTTT TTTTTTCCTTCATAA (A10)
BD_010 (MSS)	30	AGATCTCAGGTCCCACACCCTTAAGAGAAGAAAACTCACC TTCTGAGAAGATGATGTTGTCATTGCACTCATCAGAGCTA CAGGAACACATGAAGAAAGTCTCACCAGGCTTTTTTTTTTTC CTTCATAA (A10)

EXAMPLE VIII - Detection of TGFbR2 a10→a9 frameshift mutation at different annealing temperatures

5 i) Temperature gradient PCR 1 (parameter adjustment)

This experiment was for the purpose of establishing a range of temperatures at which the primer pair is effective in annealing only to the mutant DNA template, and not the wild-type DNA template.

10 Synthetic DNA (mutant (F1) and wildtype (F2)) was used as the template for PCR, with primers P2 and P4. The PCR conditions are shown in Tables 12-14, and the PCR tube layout and annealing temperature used for each tube is set out in Table 15. The annealing temperature ranged from 54°C to 60°C.

Table 12 – Tubes 1-8 PCR master mix

PCR master mix	50µL reaction		
# of samples	8		
10x Key buffer, µL	40		
10mM dNTPs, µL	8		
FW primer, µL	8	P2	
REV primer, µL	8	P4	
Template DNA, µL	8	F1	0.2 ng/µL
Taq-Polymerase, µL	2		
ddH ₂ O, µL	326		
Total, µL	400		
W\O Primer, µL	400		
Alliquot each tube, µL	50		

Table 13 – Tubes 9-16 PCR master mix

PCR master mix	50µL reaction		
# of samples	8		
10x Key buffer, µL	40		
10mM dNTPs, µL	8		
FW primer, µL	8	P2	
REV primer, µL	8	P1	
Template DNA, µL	8	F2	0.2 ng/µL
Taq-Polymerase, µL	2		
ddH ₂ O, µL	326		
Total, µL	400		
W\O Primer, µL	400		
Alliquot each tube, µL	50		

5

Table 14 – Thermocycling conditions

Step	Temperature	Time
Initial denaturation	95°C	2 min.
Denaturation (30 cycles)	95°C	15 sec.
Annealing (30 cycles)	54 - 60°C	15 sec.
Extension (30 cycles)	68°C	45 sec.
Final extension	68°C	5 min.
Hold	4°C	-

Table 15 – PCR tube layout and annealing temperature

Tube	Primer	DNA fragment	Buffer	Length	Temp.
1	P4	F1, mutant	Key	102bp	60°C
2	P4	F1, mutant	Key	102bp	59.7°C
3	P4	F1, mutant	Key	102bp	59°C
4	P4	F1, mutant	Key	102bp	57.8°C
5	P4	F1, mutant	Key	102bp	56.4°C
6	P4	F1, mutant	Key	102bp	55.2°C
7	P4	F1, mutant	Key	102bp	54.4°C
8	P4	F1, mutant	Key	102bp	54°C

Key buffer

Tube	Primer	DNA fragment	Buffer	Length	Temp.
9	P4	F2, wildtype	Key	102bp	60°C
10	P4	F2, wildtype	Key	102bp	59.7°C
11	P4	F2, wildtype	Key	102bp	59°C
12	P4	F2, wildtype	Key	102bp	57.8°C
13	P4	F2, wildtype	Key	102bp	56.4°C
14	P4	F2, wildtype	Key	102bp	55.2°C
15	P4	F2, wildtype	Key	102bp	54.4°C
16	P4	F2, wildtype	Key	102bp	54°C

Key buffer

Figure 13 shows the electrophoresis gels of the PCR products. It is clear that the primer-wildtype template interaction is most weakened at temperatures ranging from approximately 57.8°C and upwards in comparison with primer-mutant template. In addition, at approximately 59°C, the primer-wildtype template interaction is more or less completely disrupted. Primer-mutant template interaction remains strong, and the primer still has high affinity even at high temperatures.

ii) Temperature gradient PCR 2 (parameter adjustment)

This experiment was carried out in order to further establish a range of temperatures where the primer pair is effective in annealing only to the mutant DNA template.

As with part i) above, synthetic DNA (mutant (F1; SEQ ID NO: 20) and wildtype (F2; SEQ ID NO: 19)) was used as the template for PCR, with primers P2 and P4. The PCR conditions are shown in Tables 16-18, and the PCR tube layout and annealing temperature used for each tube is set out in Table 19. The annealing temperature ranged from 57°C to 62°C.

Table 16 – Tues 1-8 PCR master mix

PCR master mix	50µL reaction		
# of samples	8		
10x Key buffer, µL	40		
10mM dNTPs, µL	8		
FW primer, µL	8	P2	0.2 ng/µL
REV primer, µL	8	P4	
Template DNA, µL	8	F1	
Taq-Polymerase, µL	2		
ddH ₂ O, µL	326		
Total, µL	400		
W\O Primer, µL	400		
Alliquot each tube, µL	50		

Table 17 – Tubes 1-9 PCR master mix

PCR master mix	50µL reaction		
# of samples	8		
10x Key buffer, µL	40		
10mM dNTPs, µL	8		
FW primer, µL	8	P2	0.2 ng/µL
REV primer, µL	8	P1	
Template DNA, µL	8	F2	
Taq-Polymerase, µL	2		
ddH ₂ O, µL	326		
Total, µL	400		
W\O Primer, µL	400		
Alliquot each tube, µL	50		

5

Table 18 – Thermocycling conditions

Step	Temperature	Time
Initial denaturation	95°C	2 min.
Denaturation (30 cycles)	95°C	15 sec.
Annealing (30 cycles)	57 - 62°C	15 sec.
Extension (30 cycles)	68°C	45 sec.
Final extesion	68°C	5 min.
Hold	4°C	-

10

Table 19 – PCR tube layout and annealing temperature

Tube	Primer	DNA fragment	Buffer	Length	Temp.
1	P4	F1, mutant	Key	102bp	62°C
2	P4	F1, mutant	Key	102bp	61.7°C
3	P4	F1, mutant	Key	102bp	61.2°C
4	P4	F1, mutant	Key	102bp	60.2°C
5	P4	F1, mutant	Key	102bp	59°C
6	P4	F1, mutant	Key	102bp	58°C
7	P4	F1, mutant	Key	102bp	57.3°C
8	P4	F1, mutant	Key	102bp	57°C

Key buffer

Tube	Primer	DNA fragment	Buffer	Length	Temp.
9	P4	F2, wildtype	Key	102bp	62°C
10	P4	F2, wildtype	Key	102bp	61.7°C
11	P4	F2, wildtype	Key	102bp	61.2°C
12	P4	F2, wildtype	Key	102bp	60.2°C
13	P4	F2, wildtype	Key	102bp	59°C
14	P4	F2, wildtype	Key	102bp	58°C
15	P4	F2, wildtype	Key	102bp	57.3°C
16	P4	F2, wildtype	Key	102bp	57°C

Key buffer

Figure 14 shows the electrophoresis gels of the PCR products, and shows that even at temperatures lower than 57.8°C mentioned in part i) above, the primer-wildtype template interaction is disrupted and the affinity of the primer is close to non-existent. In particular, Figure 14 shows that the primer-wildtype template interaction is disrupted at 57°C. However, the primer affinity towards the mutant template remains strong in comparison under the same conditions.

EXAMPLE IX - Detection of TGFbR2 a10→a9 frameshift mutation using standard Taq (KCl) buffer

This experiment was conducted to assess the efficacy of the primers in PCR mixes comprising standard Taq buffer (KCl). Synthetic DNA (mutant (F1) and wildtype (F2)) was used as the template for PCR. Primers P1 and P4 were each used with primer P2. Tables 20 and 21 set out the PCR conditions, and the primers and buffer used for each tube is set out in Table 22.

Table 20 – PCR conditions for standard Taq buffer

PCR master mix (Taq)	50µL reaction
# of samples	4
Taq buffer 10x, µL	20
10mM dNTPs, µL	4

FW primer, μL	4	P2
REV primer, μL	4	Added separately
Template DNA, μL	4	Added separately
Taq-Polymerase, μL	1	
ddH ₂ O, μL	163	
Total, μL	200	
Total master mix, μL	192	
Alliquot each tube, μL	48	

Table 21 – Thermocycling conditions

Thermocycling conditions		
Step	Temperature	Time
Initial denaturation	95°C	30 sec.
Denaturation (25 cycles)	95°C	15 sec.
Annealing (25 cycles)	58°C	15 sec.
Extension (25 cycles)	68°C	45 sec.
Final extension	68°C	5 min.
Hold	4°C	-

5

Table 22 – Tube set-up

Tube	Primer	Template	Volume	Buffer
1	P1	F1	1 μL	Taq
2	P4	F1	1 μL	Taq
3	P1	F2	1 μL	Taq
4	P4	F2	1 μL	Taq

10 Figure 15 shows the electrophoresis gels of the PCR products, and shows that, for a high, uniform, temperature across the thermocycler the standard Taq buffer is stable and produced conditions which retained primer-mutant template affinity. Primer-wild type template affinity is lost, and interaction is disrupted to a point where minimal amount of fragments are produced.

15 EXAMPLE X - Detection of ASTE1 a11→a10 frameshift mutations

A panel of PCR primers for detection of mutated ASTE1 was designed in a similar way to the TGF β R2 primers of Example I.

Primers P11 and P12 are positive control forward and reverse primers, respectively, which anneal to both the wild type and the mutant ASTE1 sequence. These primers detect the presence of ASTE1 regardless of the presence or absence of the a11-to-a10 frameshift mutation (i.e. P11 and P12 are not mutant-specific), and ensure reliable results and that all criteria to run the PCR are met (no signal from the control = unsuccessful PCR).

The ASTE1 mutant primers were designed with mutant variant ASTE1 as the primary target, and each primer harbours the a10 microsatellite sequence. As with the TGF β R2 primers in Examples I and II, the shorter 10a sequence, rather than the wild type 11a sequence, introduces a mismatch between the wild type ASTE1 and the 3' end of the primers. By introducing one to four mismatches (randomly selected nucleotide substitutions) in the primers, 3' of the microsatellite, the mismatch repulsion between the primers and the wild type ASTE1 sequence is reinforced.

ASTE1 primers P16 and P24 were tested using a similar protocol to Example VIII, to establish a range of temperatures at which the primers are effective in annealing only to the mutant DNA template, and not the wild-type DNA template. Positive control reverse primer P12 was also used at a range of temperatures. Primer P11 was used as the positive primer in all experiments. Synthetic ASTE1 DNA fragments comprising either the wild type (a11) or the mutant (a10) microsatellites were used for the PCR set up (referred to as F4 and F3, respectively; SEQ ID NOs: 32 and 33, respectively).

The PCR master mix used for each PCR is shown in Table 23 below, while the PCR conditions are shown in Table 24. A different annealing temperature was used for each PCR, as shown in Tables 25-27 and Figures 16-18. Standard (KCl) buffer was used for all of the PCRs. P11 was used as the forward primer for all PCRs.

Table 23 – PCR master mix

PCR master mix (Taq) 50 μ L reaction		
# of samples	16	
Taq buffer 10x, μ L	80	
10mM dNTPs, μ L	16	
FW primer, μ L	16	P11
REV primer, μ L	16	Added separately
Template DNA, μ L	16	Added separately

Taq-Polymerase, μL	4
ddH ₂ O, μL	652
Total, μL	800
Total master mix, μL	768
Alliquot each tube, μL	48

Table 24 – Thermocycling conditions

Step	Temperature	Time
Initial denaturation	95°C	30 sec.
Denaturation (25 cycles)	95°C	15 sec.
Annealing (25 cycles)	Varying	15 sec.
Extension (25 cycles)	68°C	45 sec.
Final extension	68°C	5 min.
Hold	4°C	-

Table 25 – PCR annealing temperature for primer P16

P16-mutant (F3) well no.	P16-WT (F4) well no.	Annealing temp. (°C)
1	1	48
2	2	48.7
3	3	50
4	4	51.9
5	5	54.3
6	6	56.3
7	7	57.4
8	8	58

5

Table 26 – PCR annealing temperature for primer P24

P24-M (F3) well no.	P24-Wt (F4) well no.	Annealing temp. (°C)
1	1	44
2	2	44.9
3	3	46.8
4	4	49.5
5	5	52.8
6	6	55.6
7	7	57.2
8	8	58

Table 27 - PCR annealing temperature for primer P12

P12-F3 well no.	P12-F4 well no.	Annealing temp. (°C)
1	1	49
2	2	49.2
3	3	49.6
4	4	50.2
5	5	50.9
6	6	51.5
7	7	51.8
8	8	52

Results

Figures 16-18 show the results of these PCRs using different annealing temperatures. In particular, these Figures show that the primers designed based on a10 mutant ASTE1 (i.e. P16 and P24) are specific for a10 mutant ASTE1 and produce much lower, or no, PCR products when ASTE1 wild type is the template. Figure 18 shows that P12 can be used to amplify both wild type and a10 mutant ASTE1, such that it can be used as a positive control primer.

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5 Table 28 - Sequences

Primer	SEQ ID NO:	5' - 3' Direction	Sequence	Target
P1	1	Reverse	CAGTGT CATAGAGCACATTCCT	TGFbR2
P2	2	Forward	AGACAGTTTGCCATGACCC	TGFbR2
P4	3	Reverse	GTCTCACCAGGCTTTTTTTTC <u>G</u>	TGFbR2
P4.1	6	Reverse	GTCTCACCAGGCTTTTTTTT <u>CCG</u>	TGFbR2
P4.2	7	Reverse	GTCTCACCAGGCTTTTTTTT <u>ACCG</u>	TGFbR2
P5	8	Reverse	GTCTCACCAGGCTTTTTTTTCC	TGFbR2
P6	4	Reverse	GTCTCACCAGGCTTTTTTTT <u>ACG</u>	TGFbR2
P10	5	Reverse	CACCAGGCTTTTTTTT <u>ATCG</u> TTC	TGFbR2
P10.1	9	Reverse	CACCAGGCTTTTTTTT <u>ATCGTCC</u>	TGFbR2
P11	10	Forward	ATGTCCTGAGGCTAAGC	ASTE1
P12	11	Reverse	CACTGCACACACCAAGT	ASTE1
P12.1	38	Reverse	CACTGCACACACCAAGTG	ASTE1
P13	12	Reverse	GTTTCCTTCTGCCTTTTTTTT <u>ATCG</u>	ASTE1
P14	13	Reverse	GTTTCCTTCTGCCTTTTTTTT <u>GTCCG</u>	ASTE1
P15	14	Reverse	GTTTCCTTCTGCCTTTTTTTT <u>ACG</u>	ASTE1
P16	15	Reverse	GTTTCCTTCTGCCTTTTTTTT <u>AATCG</u>	ASTE1
P17	16	Reverse	GTTTCCTTCTGCCTTTTTTTT <u>ACTCG</u>	ASTE1
P24	31	Reverse	CTTTTTTTTCTGAATGTGATCT <u>CC</u>	ASTE1
P4x	39	Reverse	GTCTCACCAGGCTTTTTTTTTC <u>X</u>	TGFbR2
P16x	40	Reverse	GTTTCCTTCTGCCTTTTTTTT <u>X₁X₂TX₃</u> G	ASTE1
P24x	41	Reverse	CTTTTTTTT <u>X₄</u> TGAAT <u>X₅</u> TGATCT <u>X₆</u> C	ASTE1
Mutant TGFbR2 microsatellite target, a9 (SEQ ID NO: 17)			GGAAAAAAAAAGCCTGGTGAGAC	
Wild-type TGFbR2 microsatellite target, a10 (SEQ ID NO: 18)			GGAAAAAAAAAGCCTGGTGAGAC	

(bold underlined bases show mismatches to target sequence)

SEQ ID NO: 19 – Wild type TGFbR2 (a10) fragment; F2

5'TGCGAATGCTGGAGAACAGGAACCAGCTGCCGTTGTTAGGAACAAC TTCATGAAGGAAA
AGTATTCCAGATTGCCTTTCTGTCTGGAGGCCATATTATTCATTTATTCTCTTTCTCTCTCTC
CCTCTCCCCTCGCTTCCAATGAATCTCTTCACTCTAGGAGAAAGAATGACGAGAACATAAC
5 ACTAGAGACAGTTTGCCATGACCCCAAGCTCCCCTACCATGACTTTATTCTGGAAGATGCT
GCTTCTCCAAAGTGCATTATGAAGG**AAAAAAAAA**AGCCTGGTGAGACTTTCTTCATGTGTT
CCTGTAGCTCTGATGAGTGCAATGACAACATCATCTTCTCAGAAGGTGAGTTTTCTTCTCTT
AAGGGTGTGGGACCTGAGATCTGTGCCAATTTTTTTGTATCCTTGGTCTGCAGTGT CATAGA
GCACATTCCTCCTGTGGTGGATTGCATAC3'

SEQ ID NO: 20 – Mutant TGFbR2 (a9) fragment; F1

5'TGCGAATGCTGGAGAACAGGAACCAGCTGCCGTTGTTAGGAACAAC TTCATGAAGGAAA
AGTATTCCAGATTGCCTTTCTGTCTGGAGGCCATATTATTCATTTATTCTCTTTCTCTCTCTC
CCTCTCCCCTCGCTTCCAATGAATCTCTTCACTCTAGGAGAAAGAATGACGAGAACATAAC
15 ACTAGAGACAGTTTGCCATGACCCCAAGCTCCCCTACCATGACTTTATTCTGGAAGATGCT
GCTTCTCCAAAGTGCATTATGAAGG**AAAAAAAAA**AGCCTGGTGAGACTTTCTTCATGTGTT
CTGTAGCTCTGATGAGTGCAATGACAACATCATCTTCTCAGAAGGTGAGTTTTCTTCTCTTA
AGGGTGTGGGACCTGAGATCTGTGCCAATTTTTTTGTATCCTTGGTCTGCAGTGT CATAGAG
CACATTCCTCCTGTGGTGGATTGCATAC3' (bold section from residues 270 to 278)

SEQ ID NO: 32 – Wild type ASTE1 (a11) fragment; F4

5'AGAGTTCCAAAGAGTGAAGGCGCAGACACGGCTGGGCACAAGACTGGACTTAGACACA
GCTCACATCTTCTGT CAGTGGCAGTCCTGTCTCCAGATGGGGATGTATCTCAACCAGCTGC
TGTCCTACTCCTCTCCCAGAGCCAGACCTAACTCGACTGTACAGTGGAAGCCTGGTGCACG
25 GACTATGCCAGCAACTGCTAGCATCGACCTCTGTAGAAAGTCTCCTGAGCATATGTCCTGA
GGCTAAGCAACTTTATGAATATCTATTCAATGCCACAAGGTCATATGCCCCCGCTGAAATAT
TCCTACCAAAAAGGTAGATCAAATT**CAAAAAAAAAA**AGGCAGAAGAAACAGAATACCAGCT
GTTCTAAGAACAGAGGGGAGAACCACTGCACACACCAAGTGTTGGTATGAGGGAAACAACC
GGTTTGGGTTGTTAATGGTTGAAACTTAGAGGAACATAGTGAGGCCTCCAACATTGAATA
30 AAACTCAGTTTG3'

SEQ ID NO: 33 – Mutant ASTE1 (a10); F3

5'AGAGTTCCAAAGAGTGAAGGCGCAGACACGGCTGGGCACAAGACTGGACTTAGACACA
GCTCACATCTTCTGT CAGTGGCAGTCCTGTCTCCAGATGGGGATGTATCTCAACCAGCTGC
35 TGTCCTACTCCTCTCCCAGAGCCAGACCTAACTCGACTGTACAGTGGAAGCCTGGTGCACG
GACTATGCCAGCAACTGCTAGCATCGACCTCTGTAGAAAGTCTCCTGAGCATATGTCCTGA

GGCTAAGCAACTTTATGAATATCTATTCAATGCCACAAGGTCATATGCCCCCGCTGAAATAT
TCCTACCAAAAGGTAGATCAAATTC**AAAAAAAAAA**AGGCAGAAGAAACAGAATACCAGCTGT
TCTAAGAACAGAGGGAGAACCACTGCACACACCAAGTGTTGGTATGAGGGAAACAACCGG
TTTGGGTTGTTAATGGTTGAAACTTAGAGGAACATAGTGAGGCCTCCAACATTGAATAAAA
5 CTCAGTTTG3'

SEQ ID NO: 34 – wild type (a10) TGFBR2 (full length)

ATGGGTCGGGGGCTGCTCAGGGGCCTGTGGCCGCTGCACATCGTCCTGTGGACGCGTAT
CGCCAGCACGATCCCACCGCACGTTCAAGTCCGATGTGGAAATGGAGGCCAGAAAG
10 ATGAAATCATCTGCCCCAGCTGTAATAGGACTGCCCATCCACTGAGACATATTAATAACGA
CATGATAGTCACTGACAACAACGGTGACGTCAAGTTTCCACAACCTGTGTAAATTTTGTGATG
TGAGATTTTCCACCTGTGACAACCAGAAATCCTGCATGAGCAACTGCAGCATCACCTCCAT
CTGTGAGAAGCCACAGGAAGTCTGTGTGGCTGTATGGAGAAAGAATGACGAGAACATAAC
ACTAGAGACAGTTTGCCATGACCCCAAGCTCCCCTACCATGACTTTATTCTGGAAGATGCT
15 GCTTCTCCAAAGTGCAATTATGAAGG**AAAAAAAAAA**AGCCTGGTGAGACTTTCTTCATGTGTT
CCTGTAGCTCTGATGAGTGCAATGACAACATCATCTTCTCAGAAGAATATAACACCAGCAAT
CCTGACTTGTTGCTAGTCATATTTCAAGTGACAGGCATCAGCCTCCTGCCACCACTGGGAG
TTGCCATATCTGTCATCATCATCTTCTACTGCTACCGCGTTAACCGGCAGCAGAAGCTGAG
TTCAACCTGGGAAACCGGCAAGACGCGGAAGCTCATGGAGTTCAGCGAGCACTGTGCCAT
20 CATCCTGGAAGATGACCGCTCTGACATCAGCTCCACGTGTGCCAACAACATCAACCACAAC
ACAGAGCTGCTGCCCATTGAGCTGGACACCCTGGTGGGGAAAGGTCGCTTTGCTGAGGTC
TATAAGGCCAAGCTGAAGCAGAACACTTCAGAGCAGTTTGAGACAGTGGCAGTCAAGATCT
TTCCCTATGAGGAGTATGCCTCTTGGAAGACAGAGAAGGACATCTTCTCAGACATCAATCT
GAAGCATGAGAACATACTCCAGTTCCTGACGGCTGAGGAGCGGAAGACGGAGTTGGGGA
25 AACAATACTGGCTGATCACCGCCTTCCACGCCAAGGGCAACCTACAGGAGTACCTGACGC
GGCATGTCATCAGCTGGGAGGACCTGCGCAAGCTGGGCAGCTCCCTCGCCCGGGGGATT
GCTCACCTCCACAGTGATCACACTCCATGTGGGAGGCCCAAGATGCCCATCGTGACAGG
GACCTCAAGAGCTCCAATATCCTCGTGAAGAACGACCTAACCTGCTGCCTGTGTGACTTTG
GGCTTTCCCTGCGTCTGGACCCTACTCTGTCTGTGGATGACCTGGCTAACAGTGGGCAGG
30 TGGGAAGTGAAGATACATGGCTCCAGAAGTCCTAGAATCCAGGATGAATTTGGAGAATGT
TGAGTCCTTCAAGCAGACCGATGTCTACTCCATGGCTCTGGTGCTCTGGGAAATGACATCT
CGCTGTAATGCAGTGGGAGAAGTAAAGATTATGAGCCTCCATTTGGTTCCAAGGTGCGG
GAGCACCCCTGTGTCGAAAGCATGAAGGACAACGTGTTGAGAGATCGAGGGCGACCAGAA
ATTCCCAGCTTCTGGCTCAACCACCAGGGCATCCAGATGGTGTGTGAGACGTTGACTGAG
35 TGCTGGGACCACGACCCAGAGGCCCGTCTCACAGCCCAGTGTGTGGCAGAACGCTTCAG

TGAGCTGGAGCATCTGGACAGGCTCTCGGGGAGGAGCTGCTCGGAGGAGAAGATTCCTG
AAGACGGCTCCCTAAACACTACCAAATAG

SEQ ID NO: 35 – a9 TGFR2 (full length)

5 ATGGGTCGGGGGCTGCTCAGGGGCCTGTGGCCGCTGCACATCGTCCTGTGGACGCGTAT
CGCCAGCACGATCCCACCGCACGTTGAGAAGTCGGATGTGGAATGGAGGCCAGAAAG
ATGAAATCATCTGCCCCAGCTGTAATAGGACTGCCCATCCACTGAGACATATTAATAACGA
CATGATAGTCACTGACAACAACGGTGCAGTCAAGTTTCCACAACCTGTGTAAATTTTGTGATG
TGAGATTTTCCACCTGTGACAACCAGAAATCCTGCATGAGCAACTGCAGCATCACCTCCAT
10 CTGTGAGAAGCCACAGGAAGTCTGTGTGGCTGTATGGAGAAAGAATGACGAGAACATAAC
ACTAGAGACAGTTTGCCATGACCCCAAGCTCCCCTACCATGACTTTATTCTGGAAGATGCT
GCTTCTCCAAAGTGCATTATGAAGGAAAAAAAAAGCCTGGTGAGACTTTCTTCATGTGTTT
CTGTAGCTCTGATGAGTGCAATGACAACATCATCTTCTCAGAAGAATATAACACCAGCAATC
CTGACTTGTTGCTAG

15

SEQ ID NO: 36 – wild type (A11) ASTE1 (full length)

ATGGCCCATTCCTTTCTGTTGGTGGGAGTGGGTACGTATGTCCCTTACTCATCCGGGAAG
TATTCATACAGGTTTTGATCAAGCTGCGGGTGTGTTTTGTCCAGTGCTTTTCAGAAGCAGAT
CGGGACATTATGACACTTGCTAACCATTGGAATTGCCCTGTGTTATCATCAGATAGTGAATT
20 TTGCATTTTTGACCTGAAAACCTGGGTTTTGCCCATGAATAGCTTTTCAGTGGAAGAAATATGA
ACACTATTAAGGGCACACAAAACCTATATCCCTGCCAAATGCTTTTCCCTTGATGCATTCTGC
CATCACTTCAGCAATATGAATAAAGCTCTACTACCTCTCTTTGCGGTGCTATGTGGAAATGA
CCATGTTAATCTACCCATCATGGAGACATTCTTAAGTAAAGCGCGTCTTCCTCTTGAGCTA
CCAGTTCTAAAGGGAGGAGACACCACCGAATCCTGGGACTTCTGAATTGGTTGTCTCATT
25 TGCCAACCCTACCGAAGCACTAGATAATGTTCTGAAATACCTCCCAAAAAGGATCGAGAA
AATGTTAAGGAACCTCTCTGCTGTTCCATGGAAGAATACCAACAGTCCCAGGTGAAGCTAC
AGGACTTCTTCAGTGTTGTAATTATGTCTGTCCAGATGCCTTGAATCTTGTTTTACCAGAA
TGGGTATTAGTGGCTTTAGCTAAAGGCCAGCTATCTCCTTTTCATCAGTGATGCTTTGGTCCT
AAGACGGACCATTTCTCCACACAGGTGGAAAACATGCAGCAACCAAATGCCACAGAATA
30 TCTCAGCCCATCAGGCAAATCATCTATGGGCTTCTTTTAAATGCCTCACCATCTGGACAA
GACATCCTGGAATGCATTGCCTCCTCAGCCTCTAGCTTTTCAGTGAAGTGGAAGGATTAAT
AAAAATATCAGAACCTCAATCATTGATGCAGTAGAACTGGCCAAGGATCATTCTGACTTAAG
CAGATTGACTGAGCTCTCCTTGAGGAGGCGGCAGATGCTTCTGTTAGAAACCCTGAAGGT
GAAACAGACCATTTCTGGAGCCAATCCCTACTTCACTGAAGTTGCCATTGCTGTGAGTTGC
35 TACTGGTTGCAGCACACCGAGACCAAAGCAAAGCTACATCATCTACAATCCTTACTGCTCA
CAATGCTAGTGGGGCCCTTGATTGCCATAATCAACAGCCCTGGTAAGGAAGAGCTGCAGG

AAGATGGTGCTAAGATGTTGTATGCAGAGTTCCAAAGAGTGAAGGCGCAGACACGGCTGG
GCACAAGACTGGACTTAGACACAGCTCACATCTTCTGTCAGTGGCAGTCCTGTCTCCAGAT
GGGGATGTATCTCAACCAGCTGCTGTCCACTCCTCTCCCAGAGCCAGACCTAACTCGACT
GTACAGTGGAAGCCTGGTGCACGGACTATGCCAGCAACTGCTAGCATCGACCTCTGTAGA
5 AAGTCTCCTGAGCATATGTCCTGAGGCTAAGCAACTTTATGAATATCTATTCAATGCCACAA
GGTCATATGCCCCCGCTGAAATATTCTACCAAAGGTAGATCAAATTCAAAAAAAAAAG
GCAGAAGAAACAGAATACCAGCTGTTCTAAGAACAGAGGGAGAACCACTGCACACACCAA
GTGTTGGTATGAGGGAAACAACCGGTTTGGGTTGTTAATGGTTGAAACTTAGAGGAACAT
AGTGAGGCCTCCAACATTGAATAA

10

SEQ ID NO: 37 – a10 ASTE1 (full length)

ATGGCCCATTCCTTTCTGTTGGTGGGAGTGGGTACGTATGTCCCTTACTCATCCGGGAAG
TATTCATACAGGTTTTGATCAAGCTGCGGGTGTGTTTTGTCCAGTGCTTTTCAGAAGCAGAT
CGGGACATTATGACACTTGCTAACCATTGGAATTGCCCTGTGTTATCATCAGATAGTGACTT
15 TTGCATTTTTGACCTGAAAACCTGGGTTTTGCCCATGAATAGCTTTCAGTGGAGAAATATGA
ACACTATTAAGGGCACACAAAACCTATATCCCTGCCAAATGCTTTTCCCTTGATGCATTCTGC
CATCACTTCAGCAATATGAATAAAGCTCTACTACCTCTCTTTGCGGTGCTATGTGGAAATGA
CCATGTTAATCTACCCATCATGGAGACATTCTTAAGTAAAGCGCGTCTTCCTCTTGAGCTA
CCAGTTCTAAAGGGAGGAGACACCACCGAATCCTGGGACTTCTGAATTGGTTGTCTCATTT
20 TGCCAACCCTACCGAAGCACTAGATAATGTTCTGAAATACCTCCCAAAAAGGATCGAGAA
AATGTTAAGGAACTTCTCTGCTGTTCCATGGAAGAATACCAACAGTCCCAGGTGAAGCTAC
AGGACTTCTTCCAGTGTGGTACTTATGTCTGTCCAGATGCCTTGAATCTTGTTTACCAGAA
TGGGTATTAGTGGCTTTAGCTAAAGGCCAGCTATCTCCTTTCATCAGTGATGCTTTGGTCCT
AAGACGGACCATTCTTCCCACACAGGTGGAAAACATGCAGCAACCAAATGCCACAGAATA
25 TCTCAGCCCATCAGGCAAATCATCTATGGGCTTCTTTTAAATGCCTCACCATCTGGACAA
GACATCCTGGAATGCATTGCCTCCTCAGCCTCTAGCTTTCAGTGAAGTGGAAAGGATTAAT
AAAAATATCAGAACCTCAATCATTGATGCAGTAGAACTGGCCAAGGATCATTCTGACTTAAG
CAGATTGACTGAGCTCTCCTTGAGGAGGCGGCAGATGCTTCTGTTAGAAACCCTGAAGGT
GAAACAGACCATTCTGGAGCCAATCCCTACTTCACTGAAGTTGCCATTGCTGTCAGTTGC
30 TACTGGTTGCAGCACACCGAGACCAAAGCAAAGCTACATCATCTACAATCCTTACTGCTCA
CAATGCTAGTGGGGCCCTTGATTGCCATAATCAACAGCCCTGGTAAGGAAGAGCTGCAGG
AAGATGGTGCTAAGATGTTGTATGCAGAGTTCCAAAGAGTGAAGGCGCAGACACGGCTGG
GCACAAGACTGGACTTAGACACAGCTCACATCTTCTGTCAGTGGCAGTCCTGTCTCCAGAT
GGGGATGTATCTCAACCAGCTGCTGTCCACTCCTCTCCCAGAGCCAGACCTAACTCGACT
35 GTACAGTGGAAGCCTGGTGCACGGACTATGCCAGCAACTGCTAGCATCGACCTCTGTAGA
AAGTCTCCTGAGCATATGTCCTGAGGCTAAGCAACTTTATGAATATCTATTCAATGCCACAA

GGTCATATGCCCCCGCTGAAATATTCCTACCAAAGGTAGATCAAATTCAAAAAAAAAAGG
CAGAAGAAACAGAATACCAGCTGTTCTAAGAACAGAGGGAGAACCACTGCACACACCAAG
TGTTGGTATGAGGGAAACAACCGGTTTGGGTTGTAA

CLAIMS:

1. A primer for detection of a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule, wherein the mutation is a frameshift of the microsatellite as compared with the corresponding wild type microsatellite sequence, and wherein the primer comprises a region of at least 10 nucleotides that is complementary to the target sequence of the antisense or the sense strand of the DNA molecule containing the microsatellite having a frameshift mutation, except that the primer includes between one and four, or between one and three, nucleotides which are mismatched to the target sequence containing the mutation in the microsatellite and which are also mismatched to a corresponding sequence containing the wild type microsatellite, wherein the primer is configured to anneal across the length of the microsatellite having a frameshift mutation and to anneal to at least one nucleotide, or at least two nucleotides, flanking the 5' end of the microsatellite having a frameshift mutation and at least one nucleotide flanking the 3' end of the microsatellite having a frameshift mutation.
2. The primer of claim 1, wherein the primer consists of between 16 and 30 nucleotides.
3. The primer of claim 1 or 2, wherein at least one mismatched nucleotide is located in a position of the primer that is configured to anneal 3' downstream of the microsatellite or within the microsatellite, preferably wherein at least one mismatched nucleotide is within four nucleotides 5' upstream or 3' downstream of the 3' end of the microsatellite.
4. A kit for detection of a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule, wherein the mutation is a frameshift of the microsatellite as compared with the corresponding wild type microsatellite sequence, and wherein the kit comprises a first primer according to any one of claims 1 to 3, and a second primer, wherein the second primer is configured to anneal to the target sequence 3' downstream of the microsatellite on the opposite strand of the DNA molecule to the strand on which the first primer is configured to anneal.
5. The kit of claim 4, wherein the kit further comprises:
 - a third primer, wherein the third primer is configured to anneal 5' upstream of the region to which the second primer is configured to anneal, and wherein the third primer

is configured to anneal to the same strand as the first primer and the opposite strand to the second primer, or

another control primer pair, preferably wherein the sequence containing the wild type microsatellite consists of the sequence defined in SEQ ID NO: 19 or SEQ ID NO: 32, and preferably the third primer comprises the sequence defined by SEQ ID NO: 1, SEQ ID NO: 11 or SEQ ID NO: 38.

6. A primer for DNA amplification comprising the sequence defined by any one of SEQ ID NO: 3, 4, 5, 7, 15, 31, 39, 40 or 41.

7. A method for detecting a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule, wherein the mutation is a frameshift of the microsatellite as compared with the corresponding wild type microsatellite sequence, and the method comprising:

a) providing a first aliquot of a sample comprising human DNA,

b) adding to the first aliquot the necessary components for DNA amplification, a first primer and a second primer;

wherein the first primer is suitable for detection of a mutation in a microsatellite contained in a target sequence of a double stranded DNA molecule and comprises a region of nucleotides that is complementary to the target sequence containing the microsatellite having a frameshift mutation except for between one and four, or between one and three, nucleotides which are mismatched to the target sequence containing the microsatellite having a frameshift mutation and which are also mismatched to a corresponding sequence containing the wild type microsatellite and wherein the first primer is configured to anneal across the length of the microsatellite having a frameshift mutation and to anneal to at least one nucleotide flanking the 3' end of the microsatellite having a frameshift mutation and at least one nucleotide, or at least two nucleotides, flanking the 5' end of the microsatellite having a frameshift mutation;

and wherein the second primer is configured to anneal to the target sequence 3' downstream of the microsatellite, to form a first reaction mix;

wherein the first primer is configured to anneal to the sense strand of the DNA molecule and the second primer is configured to anneal to the antisense strand of the DNA molecule, or the first primer is configured to anneal to the antisense strand of the

DNA molecule and the second primer is configured to anneal to the sense strand of the DNA molecule,

- c) carrying out DNA amplification on the first reaction mix,
- d) detecting the presence of the frameshift mutation in the sample when an amplification product is produced from the DNA amplification of the first reaction mix.

8. The method of claim 7, further comprising:

- a) also adding to the first aliquot at step b) of the method either:
 - I) the second primer and a third primer, wherein the third primer is configured to anneal 5' upstream of the region to which the second primer is configured to anneal, and wherein the third primer is configured to anneal to the same strand as the first primer and the opposite strand to the second primer or
 - II) a control primer pair, to form the first reaction mix,and detecting that DNA amplification has been carried out successfully when an amplification product is produced from the DNA amplification using the second and third primers or the control primer pair; or
- b) providing a second aliquot of the sample comprising human DNA and adding to the second aliquot the necessary components for DNA amplification and either:
 - I) the second primer and a third primer, wherein the third primer is configured to anneal 5' upstream of the region to which the second primer is configured to anneal, and wherein the third primer is configured to anneal to the same strand as the first primer and the opposite strand to the second primer or
 - II) a control primer pair, to form a second reaction mix, andcarrying out DNA amplification on the second reaction mix, and detecting that DNA amplification has been carried out successfully when an amplification product is produced from the DNA amplification of the second reaction mix.

9. The method of claims 7 or 8, wherein DNA amplification is polymerase chain reaction (PCR), wherein the PCR comprises a plurality of cycles of denaturation, annealing and extension.

10. The method of claim 9, wherein step d) further comprises running the product of the PCR reaction on a gel and visualising a band to confirm that DNA amplification has been successful, preferably wherein the method further comprises step f) cutting out the band for DNA sequencing.

11. The kit of claim 4 or 5, or the method of any one of claims 7 to 10, wherein:

the frameshift mutation is in a microsatellite in the TGFBR2 gene and the target sequence comprises the sequence according to residues 270 to 278 of SEQ ID NO: 19, and wherein the first primer comprises the sequence defined by any one of SEQ ID NO: 3, 4, 5, 7 or 39 and/or the second primer comprises the sequence defined by SEQ ID NO: 2; or

the frameshift mutation is in a microsatellite in the ASTE1 gene and the target sequence comprises the sequence according to residues 328-337 of SEQ ID NO: 33, and wherein the first primer comprises the sequence defined by SEQ ID NO: 15, 31, 40 or 41, and/or the second primer comprises the sequence defined by SEQ ID NO: 10.

12. The method of claim 8, wherein:

the microsatellite is in a TGFBR2 gene, the sequence comprising the wild type microsatellite comprises the sequence according to SEQ ID NO: 19, and wherein the third primer comprises the sequence defined by SEQ ID NO: 1; or

the microsatellite is in an ASTE1 gene, the sequence comprising the wild type microsatellite comprises the sequence according to SEQ ID NO: 32, and wherein the third primer comprises the sequence defined by SEQ ID NO: 11 or SEQ ID NO: 38.

13. A method of diagnosing a disease associated with a frameshift mutation in a microsatellite, comprising carrying out the method according to any one of claims 7 to 12.

14. The method of claim 7, further comprising determining that a patient suffering from a disease or disorder associated with a frameshift mutation is suitable for a treatment targeting said frameshift mutation if the frameshift mutation is detected in step d) in a sample from the patient, preferably wherein the disease or disorder associated with a frameshift mutation is a cancer, and more preferably: wherein the cancer is colorectal cancer or gastric cancer, and the treatment targeting the frameshift mutation is FMPV-1, and the frameshift mutation is in a microsatellite in the TGFBR2 gene and comprises the sequence according to residues 270 to 278 of SEQ ID NO: 19, or the cancer is endometrial cancer or gastric cancer and the treatment targeting the frameshift mutation is FMPV-2, and the frameshift mutation is in a microsatellite in the ASTE1 gene and comprises the sequence according to residues 328 to 337 of SEQ ID NO: 33.

15. The method of claim 14, wherein:

the microsatellite is in a TGFBR2 gene, the sequence comprising the wild type microsatellite comprises the sequence according to SEQ ID NO: 19, and wherein the third primer comprises the sequence defined by SEQ ID NO: 1, preferably wherein the first primer comprises the sequence defined by any one of SEQ ID NO: 3, 4, 5, 7 or 39, and/or the second primer comprises the sequence defined by SEQ ID NO: 2; or

the microsatellite is in a ASTE1 gene, the sequence comprising the wild type microsatellite comprises the sequence according to SEQ ID NO: 32, and wherein the third primer comprises the sequence defined by SEQ ID NO: 11 or SEQ ID NO: 38, preferably wherein the first primer comprises the sequence defined by SEQ ID NO: 15, 31, 40 or 41 and/or the second primer comprises the sequence defined by SEQ ID NO: 10.

16. The method of any one of claims 7 to 15, wherein the sample is a liquid biopsy comprising cell free DNA, preferably wherein the liquid biopsy is plasma.

17. The method of any one of claims 7 to 16, wherein the DNA amplification is PCR, and the PCR is carried out in high stringency conditions, optionally wherein the high stringency conditions comprise at least one of:

a) carrying out the annealing step of PCR at a temperature that is at least 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C or 10°C higher than the recommended annealing temperature of the reaction;

b) carrying out the annealing step of PCR for only 30 seconds, preferably 15 seconds, per cycle;

c) carrying out the DNA amplification in a buffer concentration that is less than 0.1X, 0.2X, 0.3X, 0.4X, 0.5X, 0.6X or 0.75X;

d) carrying out the DNA amplification in a buffer comprising ammonium ions; and

e) performing 30 or fewer, 25 or fewer, 20 or fewer, 15 or fewer, or 10 or fewer, cycles of PCR.

Fig. 1A

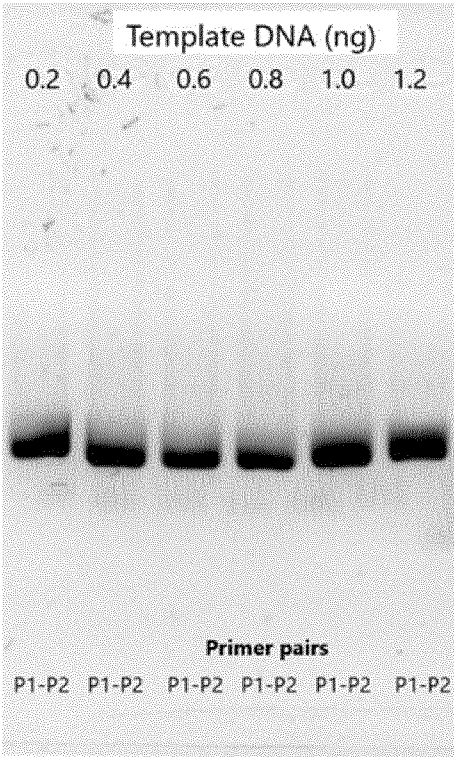
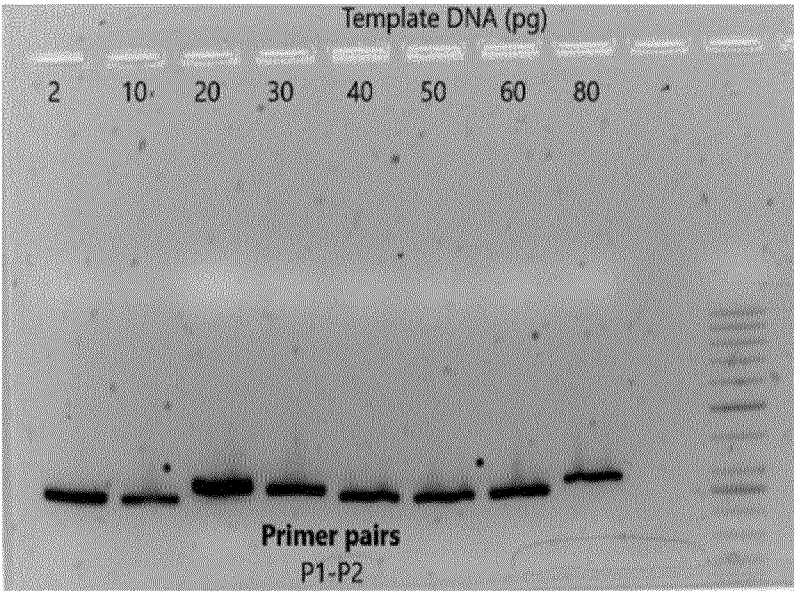
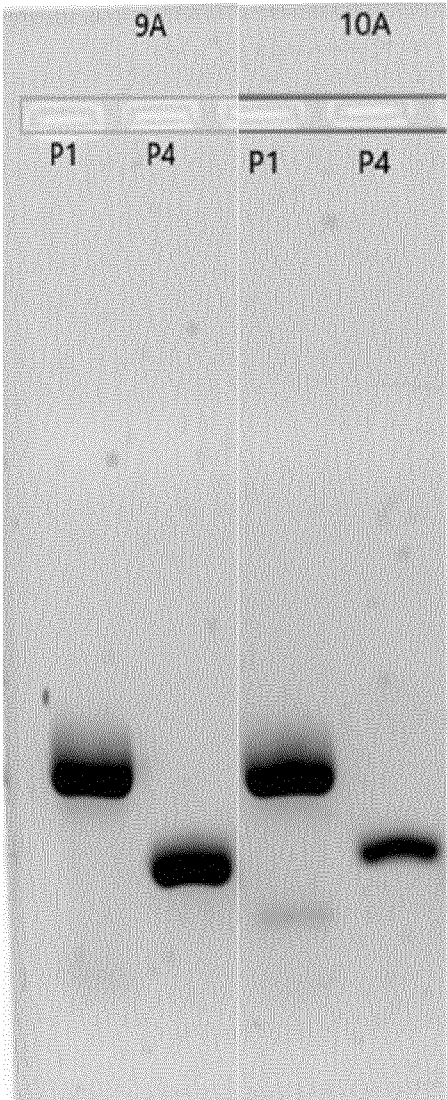


Fig. 1B



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Fig. 2



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Fig. 3

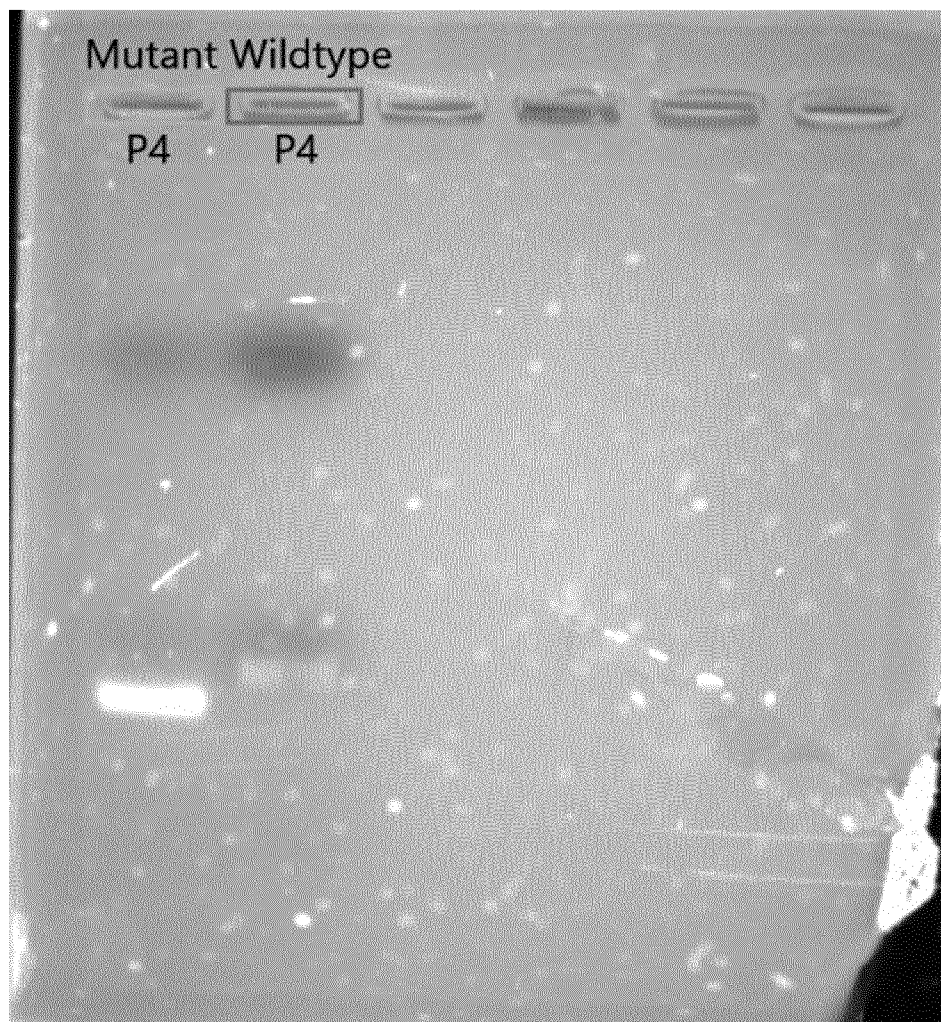


Fig. 4

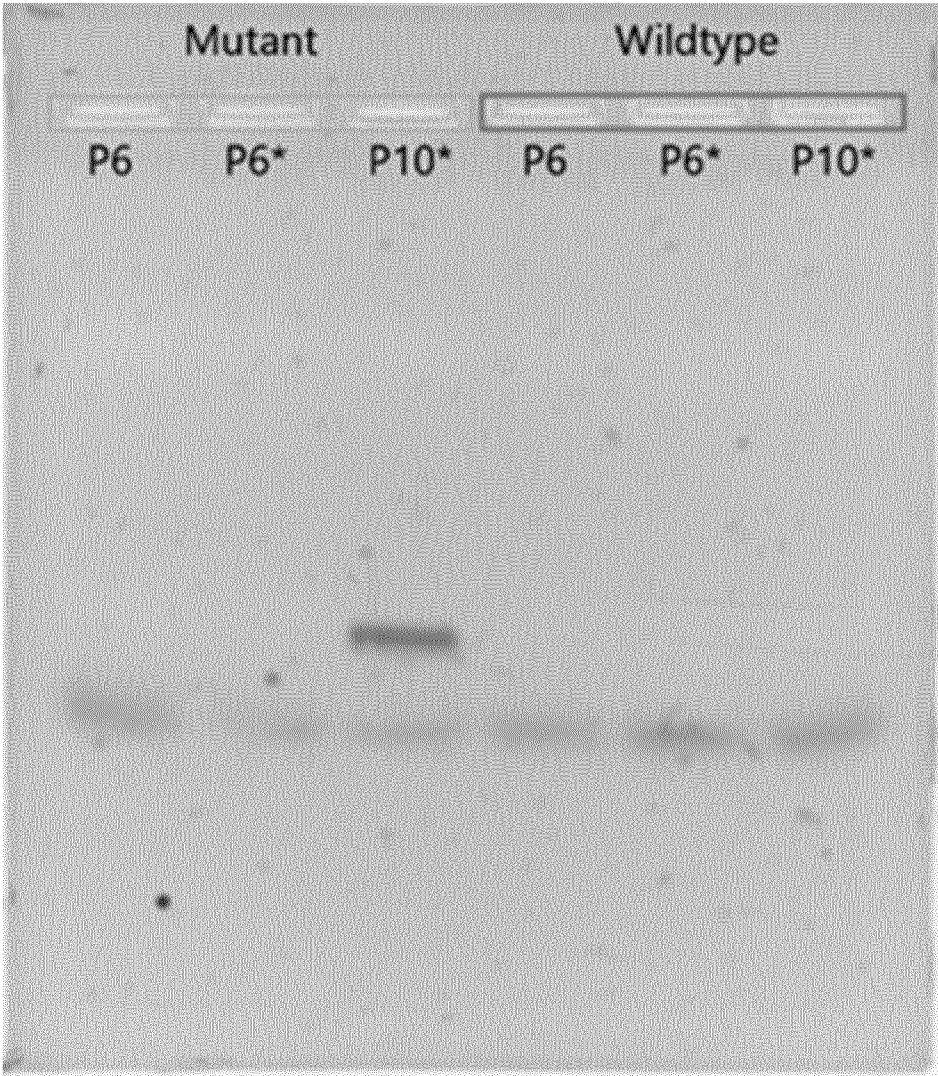


Fig. 5A

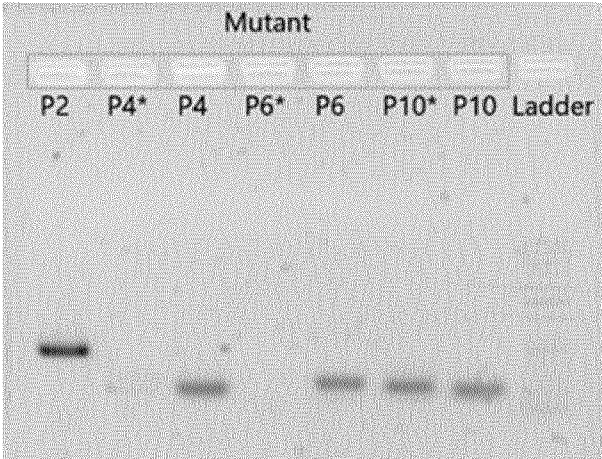


Fig. 5B

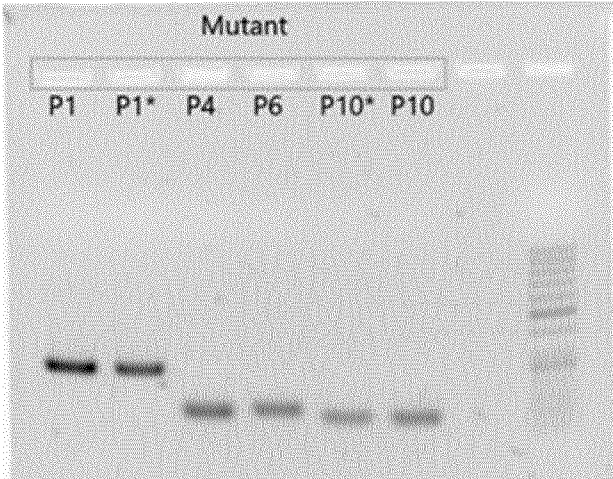


Fig. 5C



Fig. 6A

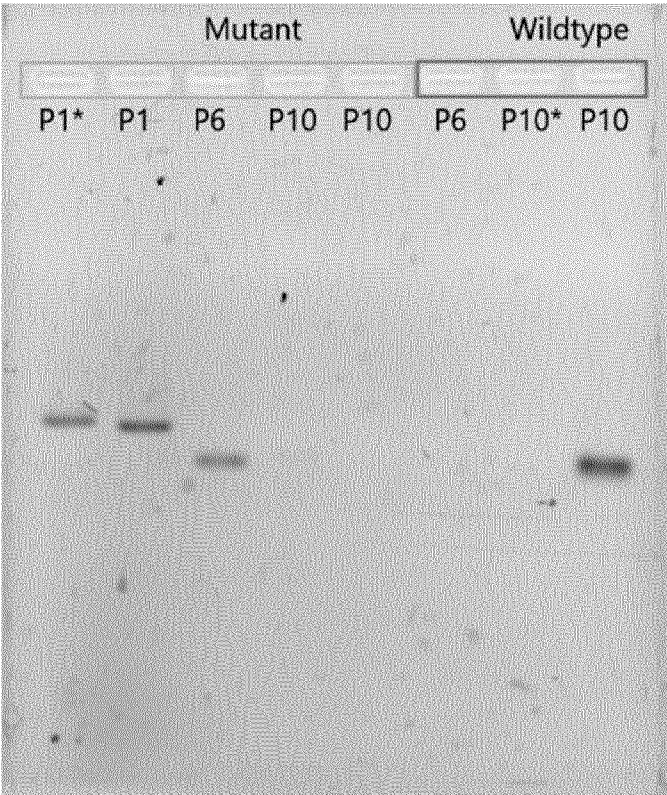
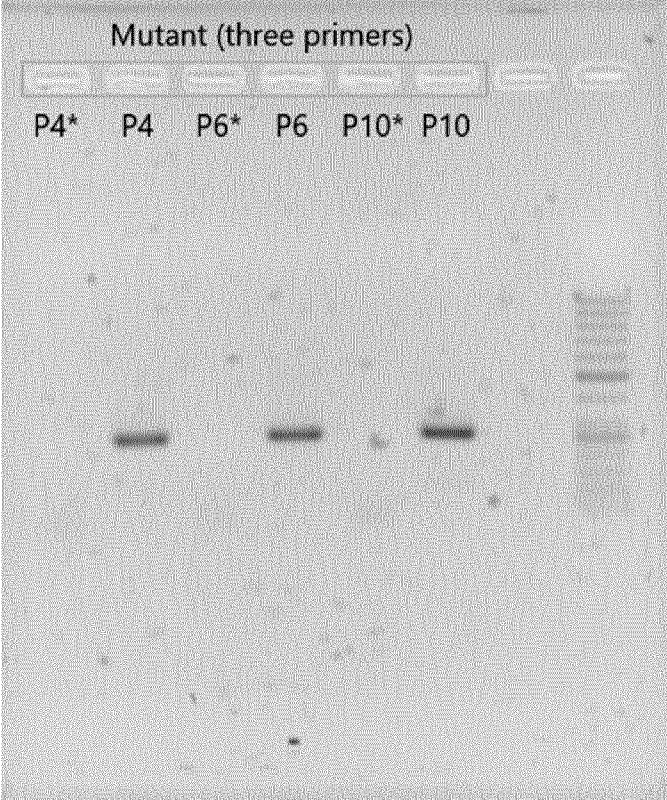


Fig. 6B



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Fig. 7

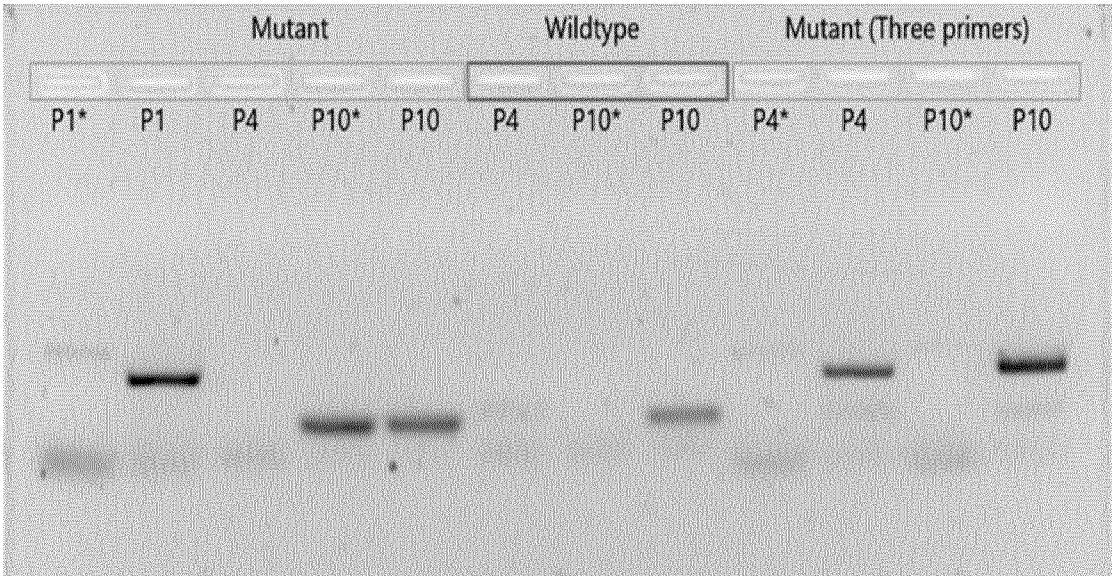
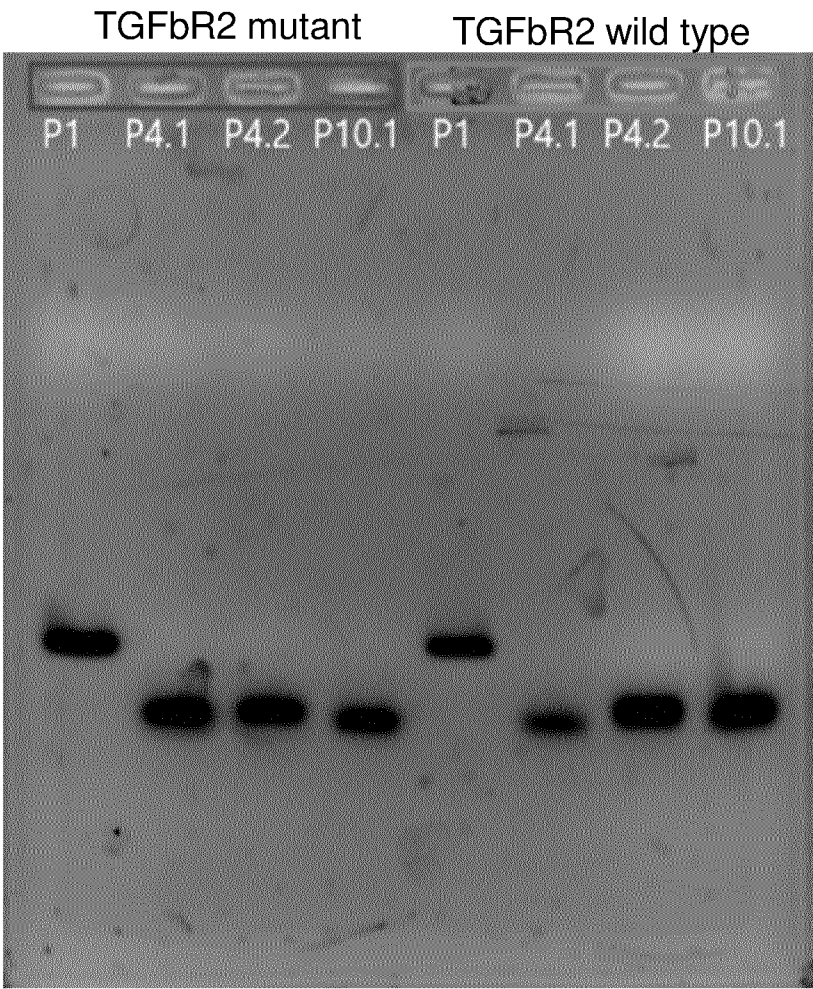


Fig. 8



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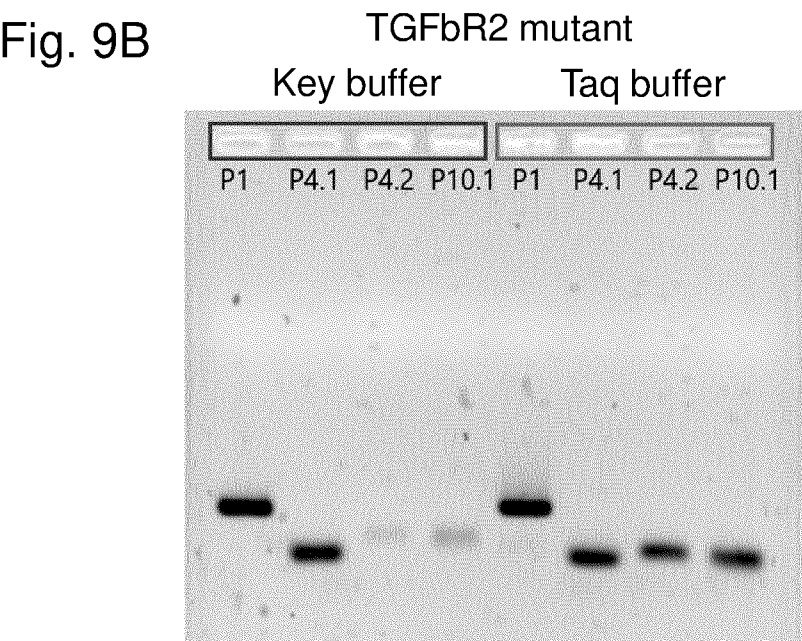
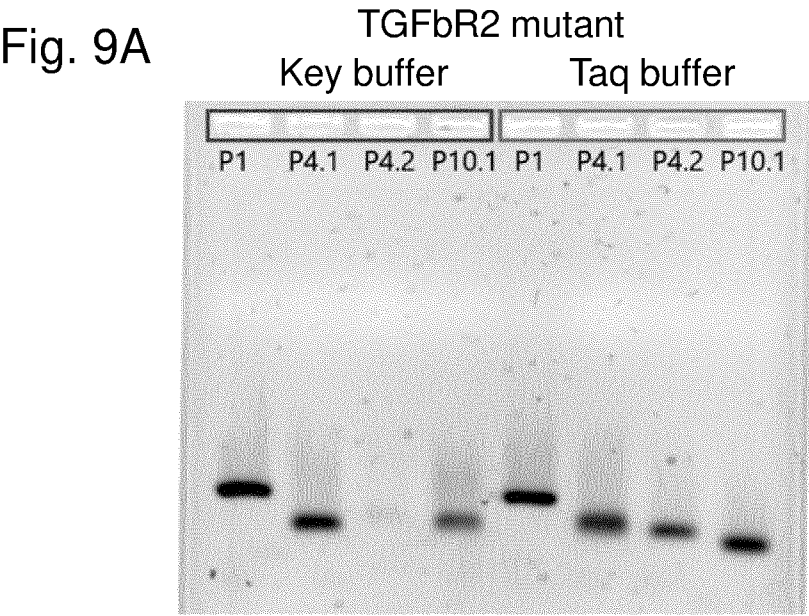


Fig. 10A

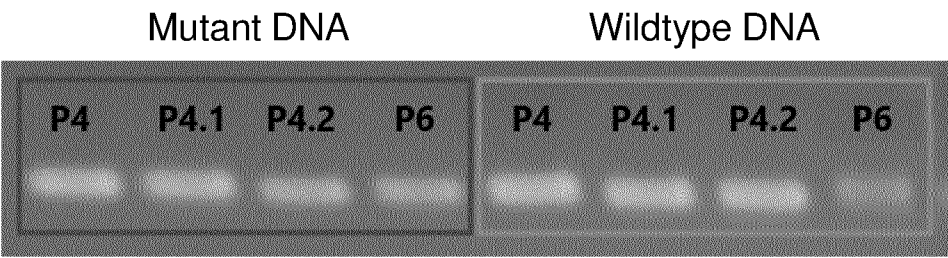


Fig. 10B

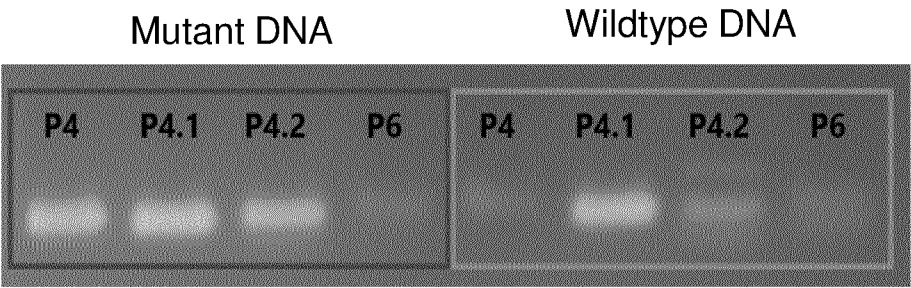


Fig. 11A

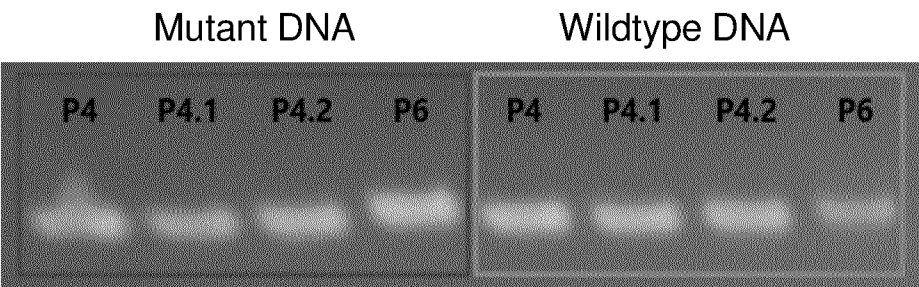
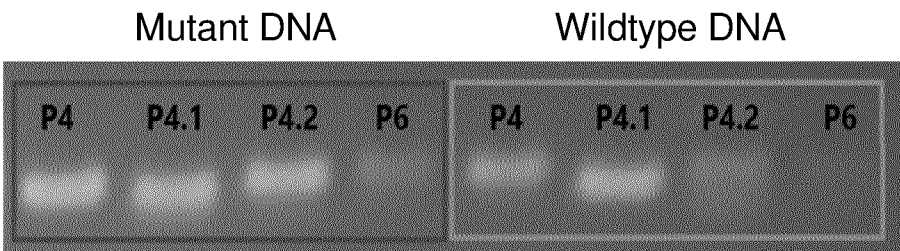


Fig. 11B



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Fig. 12

	Patient	Primer pair		Microsatellite length	
		p4 ¹	CP ²	a9	a10
MSI	BD_001	yes	yes	+	-
	BD_002	yes	yes	+	-
	BD_003	yes	yes	+	-
	BD_004	yes	yes	+	-
	BD_005	yes	yes	+	-
MSS	BD_006	No	Yes	-	+
	BD_007	No	Yes	-	+
	BD_008	No	Yes	-	+
	BD_009	No	Yes	-	+
	BD_010	No	Yes	-	+

Fig. 13

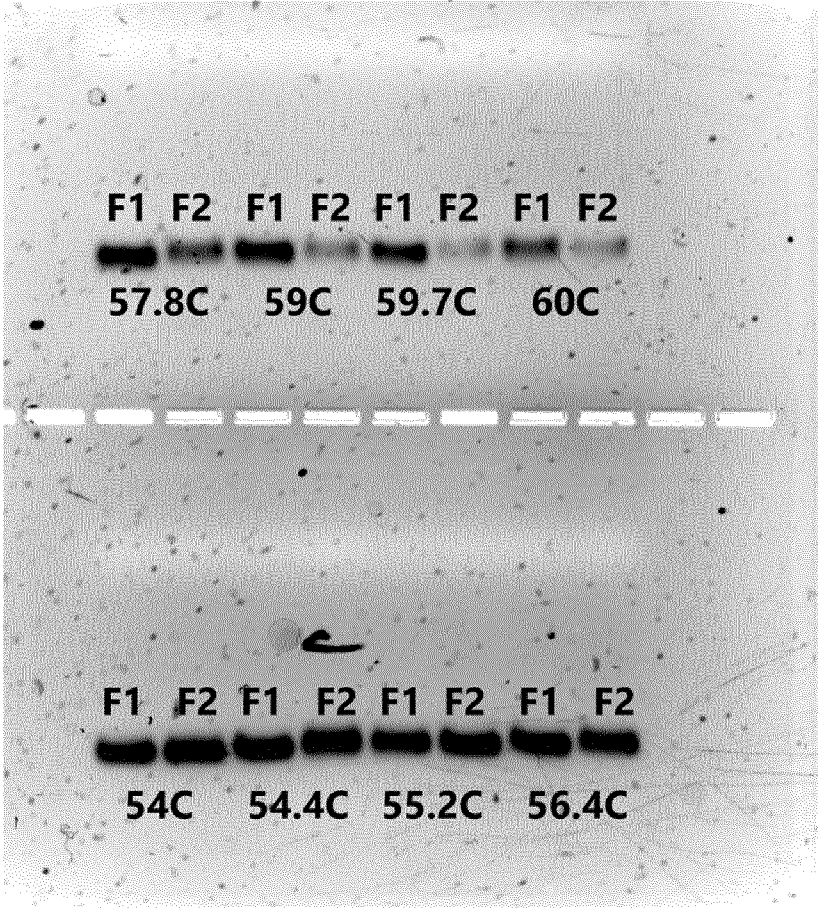


Fig. 14

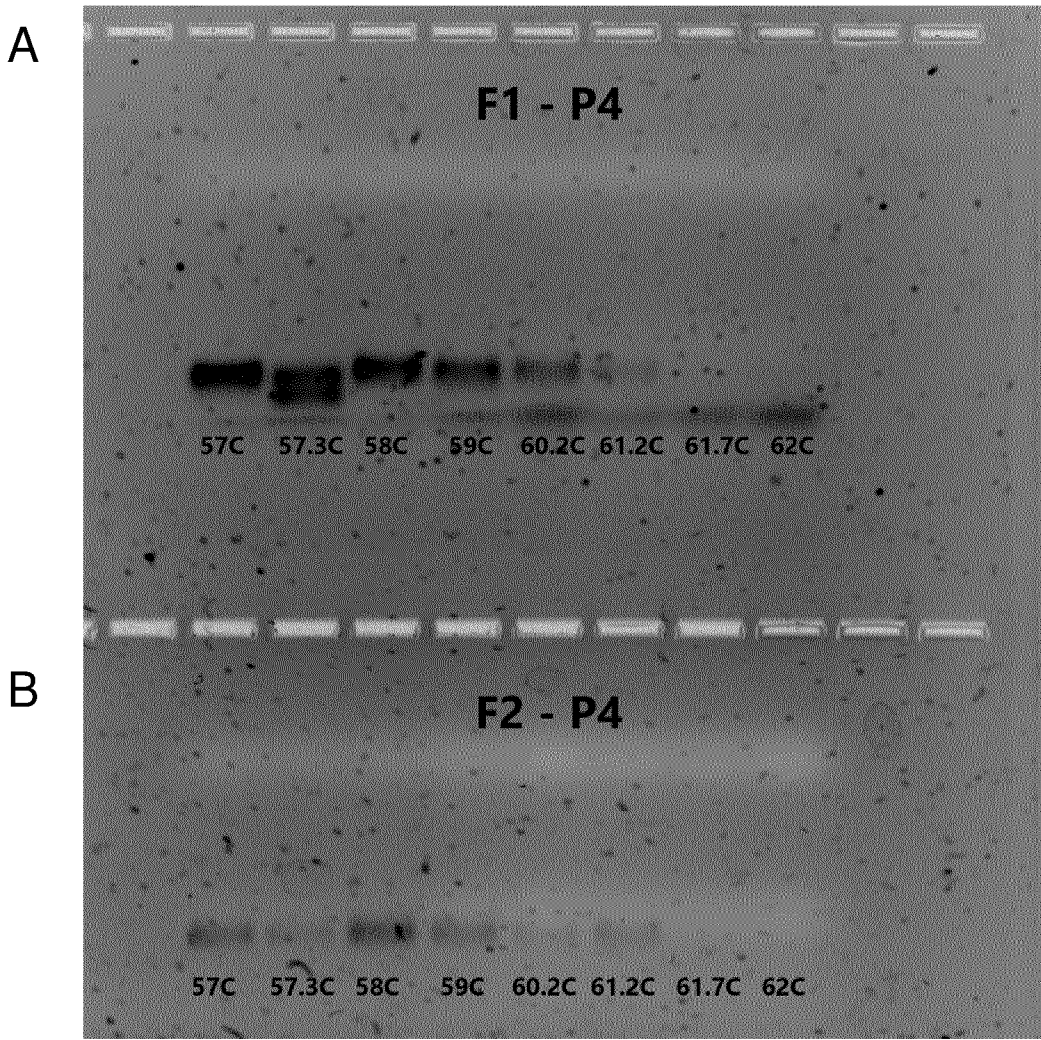


Fig. 15

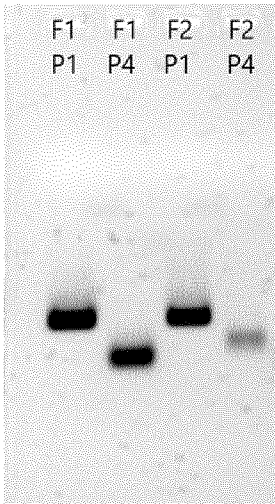


Fig. 16

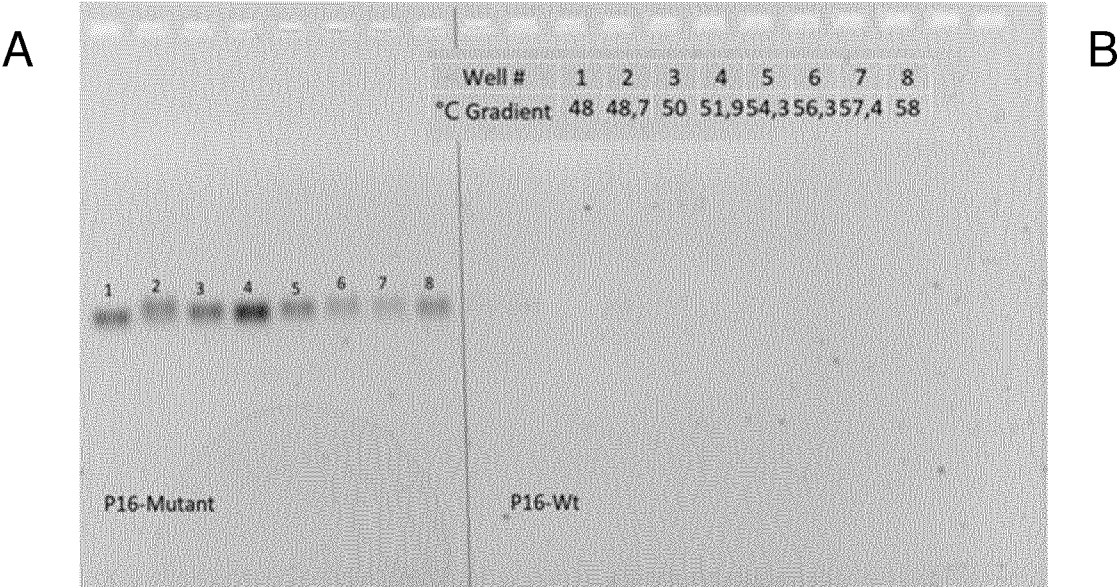


Fig. 17

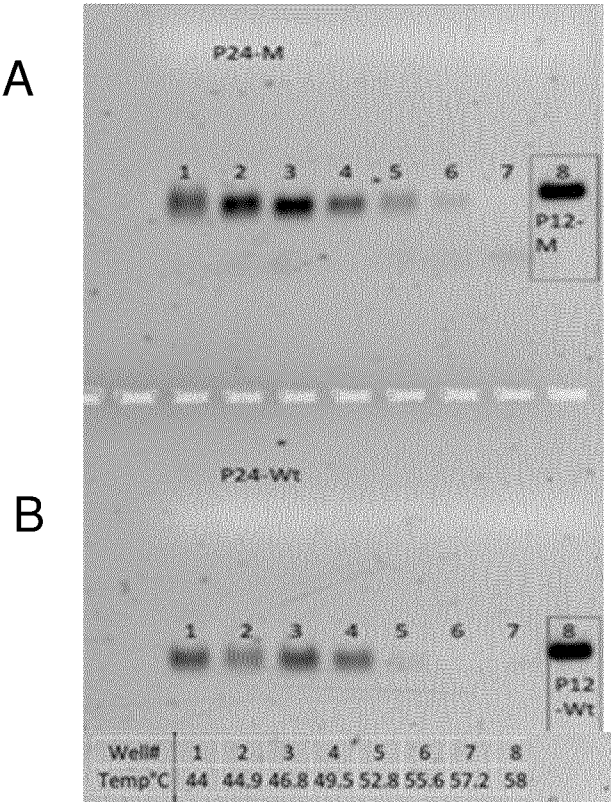
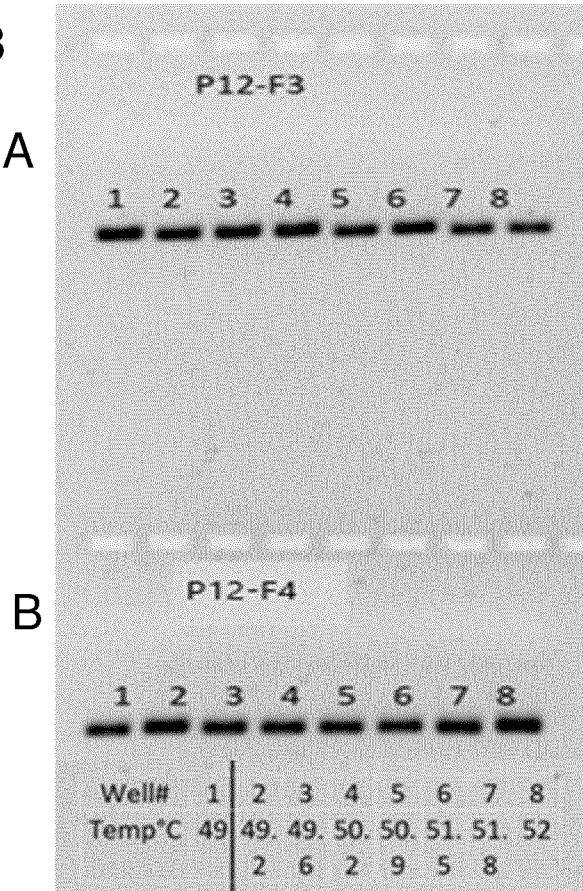


Fig. 18



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2022/069276

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/6827 C12Q1/6858

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, Sequence Search**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 109 609 617 A (HEFEI DAHUI GENE TECH CO LTD ET AL.) 12 April 2019 (2019-04-12) claim 1 -----	1
X	DATABASE Geneseq [Online] 26 July 2007 (2007-07-26), "Human MMP7 -181 A/G DNA amplifying PCR primer, SEQ ID: 89.", XP055867654, retrieved from EBI accession no. GSN:AGB10631 Database accession no. AGB10631 the whole document ----- -/--	1



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 October 2022

Date of mailing of the international search report

20/10/2022

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2

NL - 2280 HV Rijswijk

Tel. (+31-70) 340-2040,

Fax: (+31-70) 340-3016

Authorized officer

Costa Roldán, Nuria

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2022/069276

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 2 850 185 A1 (RANA THERAPEUTICS INC [US]; GEN HOSPITAL CORP DBA [US]) 25 March 2015 (2015-03-25) page 117 -----	1
Y	AKIYAMA ET AL: "Transforming growth factor beta type II receptor gene mutations in adenomas from hereditary nonpolyposis colorectal cancer", GASTROENTEROLOGY, ELSEVIER INC, US, vol. 112, no. 1, 1 January 1997 (1997-01-01), pages 33-39, XP005178557, ISSN: 0016-5085, DOI: 10.1016/S0016-5085(97)70216-6 page 38 - page 39 -----	1-5, 7-17
Y	WO 2004/003173 A2 (UNIV STATE CLEVELAND [US]; GUO BAOCHUAN [US]) 8 January 2004 (2004-01-08) page 21 -----	1-5, 7-17
A	WO 2018/037231 A1 (THE UNIV OF NEWCASTLE UPON TYNE [GB]) 1 March 2018 (2018-03-01) page 31, line first; claim 1 -----	1-17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2022/069276

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2022/069276

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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			WO 2018037231 A1 01-03-2018