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(54) Title: PRIMER SET, KIT AND METHODS THEREOF

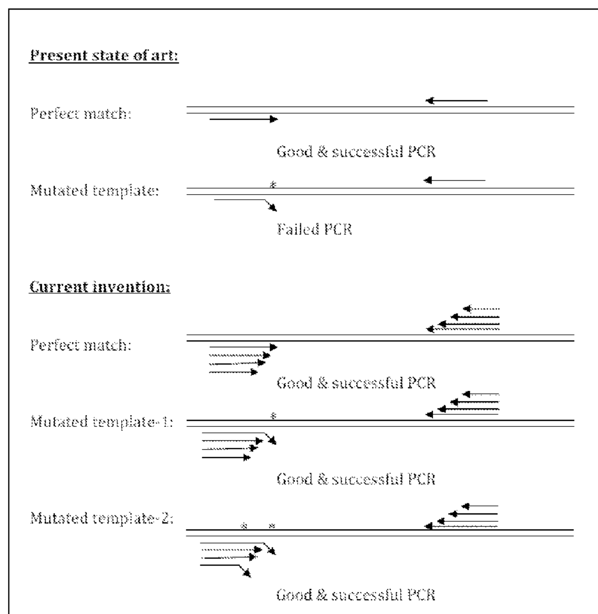

FIGURE 2

(57) Abstract: The present disclosure provides for a primer designing for amplification of templates with unpredicted sequence variations. A major hurdle in designing primers for amplifications from microbial genomes is the presence of mutations, also called Single Nucleotide Polymorphisms (SNPs) among the microbial strains. One set of primer designed for PCR based detection of a particular strain of organism might fail to amplify another strain. To overcome this, multiple strains (nation-wide or globally) are sequenced and consensus / wobble primers are made. Furthermore, long-term validation programs continue as the microbes keep mutating continuously. The present disclosure proposes a novel concept to design primers that can positively and accurately amplify targets from all strains/mutants of a microbe or an organism.

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"PRIMER SET, KIT AND METHODS THEREOF"

TECHNICAL FIELD

The present disclosure provides for a primer designing technique for amplification of templates with unpredicted sequence variations. The present disclosure proposes a novel concept to design primers that can positively and accurately amplify targets from all strains/mutants of a microbe or an organism.

BACKGROUND OF THE DISCLOSURE

Major hurdle in PCR amplification of any microbial genome is sequence variations (SNPs) within strains. Thus, a set of primers designed based on data obtained from one strain / isolate might fail to amplify the same target from another strain / isolate. To overcome these problems, large validation exercises are taken up to develop consensus primers.

Currently, molecular diagnostic kits are available that rely on detection of presence of microbes / templates by PCR. Primers are designed and validated for the templates available across the country / region. Validations are required to ensure that there are no mismatches between the primer sequence and the primer annealing sequence within templates available in the Country / region. Further, even the kits developed and validated do not perform after few years as the organisms mutate over time.

Some of the limitations of the existing technology:

1. A library of sequence data has to be generated and validated for developing consensus primers.
2. The microorganisms keep evolving / mutating continuously and more SNPs are introduced in their genome and thus, the consensus primers fail to amplify on PCRs over time.
3. Mismatch(es) at 3' end of primer annealing site (because of mutations introduced) can reduce PCR efficiency by 3-logs. (Reference: "Quantification of the Detrimental Effect of a Single Primer-Template Mismatch by Real-Time PCR

Using the 16S rRNA Gene as an Example"; D. Bru, F. Martin-Laurent, and L. Philippot; APPLIED AND ENVIRONMENTAL MICROBIOLOGY; Mar. 2008, p. 1660–1663").

5 **STATEMENT OF THE DISCLOSURE**

Accordingly, the present disclosure relates to a primer set comprising plurality of primers having a maximum length N, ranging from about 19 to about 45 nucleotides, wherein each primer has a length selected from a group comprising N, N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, N-11, N-12, N-13, N-14, N-15, N-16, N-17, N-18, N-19, N-
10 20, N-21, N-22, N-23, N-24, N-25 and N-26 or any combination thereof, wherein the primers are corresponding to a consensus sequence flanking a region of a target nucleotide sequence to be amplified, said region having mutation or suspected of having mutation at N^{th} position; a kit for amplifying a region of target nucleotide sequence having mutation or suspected of having mutation at N^{th} position, said kit having a primer
15 set comprising plurality of primers having a maximum length N, ranging from about 19 to about 45 nucleotides, wherein each primer has a length selected from a group comprising N, N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, N-11, N-12, N-13, N-14, N-15, N-16, N-17, N-18, N-19, N-20, N-21, N-22, N-23, N-24, N-25 and N-26 or any combination thereof, wherein the primers are corresponding to a consensus sequence
20 flanking said region, optionally along with DNA polymerase and nucleic acid amplification reagent selected from a group comprising magnesium chloride, dNTPs and buffer or any combination of reagents thereof; a method of obtaining a primer set comprising plurality of primers having a maximum length N, ranging from about 19 to about 45 nucleotides, wherein each primer has a length selected from a group comprising
25 N, N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, N-11, N-12, N-13, N-14, N-15, N-16, N-17, N-18, N-19, N-20, N-21, N-22, N-23, N-24, N-25 and N-26 or any combination thereof, said method comprising acts of: (a) identifying a region for amplification having mutation or suspected of having mutation at N^{th} position in a target nucleotide sequence, followed by observing a consensus sequence flanking the said N^{th}
30 position, and (b) synthesizing plurality of primers corresponding to the consensus sequence, wherein length of the primers range from about 19 to about 45 nucleotides to

obtain said primer set; and a method of amplifying a region of target nucleotide sequence having mutation or suspected of having mutation at N^{th} position, said method comprising act of subjecting the target nucleotide sequence to the reaction mixture as claimed above, for amplifying the said region.

5

BRIEF DESCRIPTION OF ACCOMPANYING FIGURES

In order that the disclosure may be readily understood and put into practical effect, reference will now be made to exemplary embodiments as illustrated with reference to the accompanying figures. The figure together with a detailed description below, are 10 incorporated in and form part of the specification, and serve to further illustrate the embodiments and explain various principles and advantages, in accordance with the present disclosure where:

Figure 1 shows 4 strains which are indicated with minor variations / mutations within the region of primer annealing.

15 **Figure 2** shows a comparative analysis between the present disclosure and prior art.

Figure 3 shows Amplicon from conventional PCR and PCR based on the present disclosure.

Figure 4 shows the results of PCR when the conventional primers are used (a) and the results of PCR when the primer set designed by the present disclosure are used (b).

20 **Figure 5** shows the results of PCR when the conventional primers (fluorescent labeled) are used (a) and the results of PCR when the primer set (also fluorescent labeled) designed by the present disclosure are used (b).

DETAILED DESCRIPTION

25 The present disclosure relates to a primer set comprising plurality of primers having a maximum length N, ranging from about 19 to about 45 nucleotides, wherein each primer has a length selected from a group comprising N, N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, N-11, N-12, N-13, N-14, N-15, N-16, N-17, N-18, N-19, N-20, N-21, N-22, N-23, N-24, N-25 and N-26 or any combination thereof, wherein the primers are 30 corresponding to a consensus sequence flanking a region of a target nucleotide sequence

to be amplified, said region having mutation or suspected of having mutation at N^{th} position.

In an embodiment of the present disclosure, one of the primers in the set is having a length N and remaining primers in the set are having a length ranging from about N-1 to
5 about N-26 in any order thereof.

The present disclosure further relates to a kit for amplifying a region of target nucleotide sequence having mutation or suspected of having mutation at N^{th} position, said kit having a primer set comprising plurality of primers having a maximum length N, ranging from
10 about 19 to about 45 nucleotides, wherein each primer has a length selected from a group comprising N, N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, N-11, N-12, N-13, N-14, N-15, N-16, N-17, N-18, N-19, N-20, N-21, N-22, N-23, N-24, N-25 and N-26 or any combination thereof, wherein the primers are corresponding to a consensus sequence flanking said region, optionally along with DNA polymerase and nucleic acid
15 amplification reagent selected from a group comprising magnesium chloride, dNTPs and buffer or any combination of reagents thereof;

In an embodiment of the present disclosure, the kit is a reaction mixture used for amplifying said region of the target nucleotide sequence.

20

In another embodiment of the present disclosure, the amplification is carried out by technique selected from a group comprising Conventional Polymerase Chain Reaction, HotStart Polymerase Chain Reaction, Real Time Polymerase Chain Reaction and Isothermal Amplifications.

25

In yet another embodiment of the present disclosure, the mutation is selected from a group comprising unpredicted sequence variations, SNPs, deletions, alternate splice products, microsatellites and gene rearrangements or any combination thereof.

30 The present disclosure further relates to a method of obtaining a primer set comprising plurality of primers having a maximum length N, ranging from about 19 to about 45

nucleotides, wherein each primer has a length selected from a group comprising N, N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, N-11, N-12, N-13, N-14, N-15, N-16, N-17, N-18, N-19, N-20, N-21, N-22, N-23, N-24, N-25 and N-26 or any combination thereof, said method comprising acts of: (a) identifying a region for amplification having mutation or suspected of having mutation at N^{th} position in a target nucleotide sequence, followed by observing a consensus sequence flanking the said N^{th} position, and (b) synthesizing plurality of primers corresponding to the consensus sequence, wherein length of the primers range from about 19 to about 45 nucleotides to obtain said primer set.

The present disclosure further relates to a method of amplifying a region of target nucleotide sequence having mutation or suspected of having mutation at N^{th} position, said method comprising act of subjecting the target nucleotide sequence to the reaction mixture as claimed above, for amplifying the said region.

In an embodiment of the present disclosure, the amplification with said reaction mixture provides amplified product of the mutated region.

In another embodiment of the present disclosure, the amplified product is detected by techniques selected from a group comprising Agarose based detection, spectrophotometric detection and fluorometric detection or any combination thereof.

In yet another embodiment of the present disclosure, primers of the reaction mixture amplifies said region of the target nucleotide sequence by binding to a consensus sequence flanking the region having mutation or suspected of having mutation at N^{th} position.

In still another embodiment of the present disclosure, primers of the reaction mixture anneal at same location at 3' end of the consensus sequence and at different locations at 5' end of the consensus sequence.

In still another embodiment of the present disclosure, the amplification is carried out by technique selected from a group comprising Conventional Polymerase Chain Reaction, HotStart Polymerase Chain Reaction, Real Time Polymerase Chain Reaction and Isothermal Amplifications.

5

In still another embodiment of the present disclosure, the mutation is selected from a group comprising unpredicted sequence variations, SNPs, deletions, alternate splice products, microsatellites and gene rearrangements or any combination thereof.

10 In still another embodiment of the present disclosure, the target nucleotide sequence is selected from a group comprising prokaryotic and eukaryotic genomic sequence or a combination thereof.

The present disclosure provides for a novel concept to design primers for amplification of 15 templates with probable variations/ mutations. Primers are designed from data available from one strain / isolate that can positively and accurately amplify targets from all related strains irrespective of sequence variations. Further, development of series of primers and large-scale validation experiments will not be required and the PCR amplifications will work in spite of evolutionary changes or mutations. Hence, the present disclosure is a 20 valuable tool to eliminate false negative cases in molecular diagnostics.

The present disclosure presents the following aspects:

1. A set of primers designed which amplifies the target sequence irrespective of whether there are mutations in the sequence where the primers anneal.
2. (25) The primers are designed based on available information in public domains.
3. The primers (more than one forward primers in combination with more than one reverse primers) are used simultaneously for single reaction.
4. All forward primers anneal at identical position in the 5'-end but end at different positions at the 3'-end.
5. (30) The primers are one or more bases smaller than each other.

6. Any one or more of the forward primer(s) would anneal on template and generate amplicons in combination with any one or more reverse primer(s).
7. The size of the amplicons is observed to be always identical irrespective of which set of the primers are used to amplify and give the amplicons.
8. The concept would make "validation" requirements redundant which would be beneficial in the following ways:
    a. No special knowledge of sequence is required.
    b. Large expenses spent on Validation would be saved.
    c. Long duration validation process can be avoided. Developmental processes will be faster.
    d. The life of the kits will be prolonged. That means, once developed, the kits will be performing equally well over the years though the microbes / template might mutate over time.

THE PRIMER DESIGNING PROTOCOL:

a) The primers are designed based on reference sequence data available.
b) Design 2 to 'N' sets of both forward and reverse primers each that anneal at identical regions on lower strand and upper strands respectively.
c) The forward primers are a combination of 2, 3 or more primers which are 1, 2, 3 or more bases smaller than each other. It is the same for the reverse primer as well.
d) The forward primers' annealing position at the 5'-end are identical but different at the 3'-end (Please refer to figure 1). It is the same for the reverse primers as well.
e) The primer-sets are mixed and PCR performed.

In an embodiment of the present disclosure, PCR always works even if there are mutations on the template within primer annealing sites. Further, the PCR products are detected by running samples on agarose gels (and run along with molecular weight markers for proper sizing) or by spectrophotometric / fluorometric detection.

POSSIBILITIES OF PRIMER SIZES / COMBINATIONS, RULES:

1. There can be more than one forward and more than one reverse primers for PCR.
2. Maximum length of the longest primer (forward & reverse): between 20 and 45 bases.
3. Minimum length of the smallest primer (forward & reverse): Between 19 and 44 bases.
4. The second longest primer (forward & reverse) size can be smaller than 45 bases and can be till the point of a consensus region.
5. Similarly, the third longest primer size (forward & reverse) can be smaller than the second longest primer and can be till the point of another consensus region.
6. All forward and reverse primers are mixed together and PCRs are performed.
7. In presence of mutations on template, while any one or more of primers fail to extend/ polymerize (because of mismatches between primer and template sequences), the other primers extend / polymerize to give PCR products. The same happens in case of mutations within both forward as well as reverse primer annealing positions.

Further, when the primers are used individually in combination of single forward and single reverse primer, the PCR reactions fail in case there are mutations in the last base (alone or in combination with other mutations spread around).

For example, if only the N-4 of Forward primer and only N-2 of Reverse primers are used in combination, the PCRs would fail in the following cases:

1. if there are mutations in 3'-end positions (where the primer ends) on the forward primer annealing region.
2. if there are mutations in 3'-end positions (where the primer ends) on the reverse primer annealing region.

The present disclosure is further elaborated with the help of following examples and associated figures. However, these examples should not be construed to limit the scope of the present disclosure. Further, as the scope of the present disclosure is non-limiting in

nature, the instant primers can be applied to various other biotechnological applications such as but not limiting to those illustrated in the following examples. Further, all the possible amplification experiments involved in all such biotechnological applications fall under the purview of the present disclosure.

5

EXAMPLES

EXAMPLE 1: Schematic representation of sequence data for a targeted part of the template genome for PCR amplification

The figure 1 depicts 4 strains which are indicated with minor variations / mutations within the region of primer annealing and demonstrates how the primers would still anneal (and thus, PCR amplify) in spite of those mutations.

10

Assume only Strain A sequence is known while Strains B, C and D are variants present in nature.

15

Based on Strain-A sequence, the following primers are designed:

Primer-1: BBB BBB BBB BBB BBB **GAC TGA CTC AGT C** Size: 28 bases (size = N)

20 Primer-2: BBB BBB BBB BBB BBB **GAC TGA CTC AG** Size: 26 bases (size = N-2)

Primer-3: BBB BBB BBB BBB BBB **GAC TGA CTC** Size: 24 bases (size = N-4)

25 Where B is a base: A, T, G or C

All these three primers are used together in the PCR reaction and the observations are as follows:

For Strain A: all the 3 primers anneal and give the product.

30 For Strain B: Only Primer-2 and Primer-3 give product since Primer-1 would fail as it has last base mismatch.

For Strain C: Only Primer-2 and Primer-3 give product since Primer-1 would fail as it has penultimate base mismatch.

For Strain D: Only Primer-1 and Primer-3 give product since Primer-2 has penultimate base mismatch.

Use of all the three sets of primers together with N, N-2 and N-4 bases in size ensures that all mutant strains are amplified successfully and accurately.

EXAMPLE 2:

In an embodiment, a wild template and a corresponding mutated template is generated in order to showcase the efficacy and success of amplification of the mutated template by the primers designed according to the present disclosure.

Wild Template:

CTCACGCTGCTCGTTGAGTTTTGATTTTGCTGTTTCAAGCTCAACACGCAGTTT
CCCTACTGTTAGCGCAATATCCTCGTTCTCCTGGTCGCGGCGTTTGATGTATT
GCTGGTTTCTTTCCCGTTCATCCAGCAGTTCCAGCACAATCGATGGTGTTACC
AATTCATGGAAAAGGTCTGCGTCAAATCCCCAGTCGTCATGCATTGCCTGCTC
TGCCGGTTCACGCTGTGCCTGAGAGTTAATTTCGCTCACTTCGAACCTCTCTG
TTTACTGATAAGTTCCAGATCCTCCTGGCAACTTGCACAAGTCCGACAACCCT
GAACGACCAGGCGTCTTCGTTCATCTATCGGATCGCCACACTCACAACAATG
AGTGGCAGATATAGCCTGGTGGTTCAGGCGGCGCATTTTTATTGCTGTGTTGC
GCTGTAATTCTTCTATTTCTGATGCTGAATCAATGATGTCTGCCATC

Mutant Template:

CTCACGCTGCTCGTTGAGTTTTGATTTTGCTGTTTCAAGCTCAACACGCAGTTT
CCCTACTGTTAGCGCAATATCCTCGTTCTCCTGGTCGCGGCGTTTGATGTATT
GCTGGTTTCTTTCCCGTTCATCCAGCAGTTCCAGCACAATCGATGGTGTTACC
AATTCATGGAAAAGGTCTGCGTCAAATCCCCAGTCGTCATGCATTGCCTGCTC
TGCCGCTTCACGCAGTGCCTGAGAGTTAATTTCGCTCACTTCGAACCTCTCTG

TTTACTGATAAGTTCCAGATCCTCCTGGCAACTTGCACAAGTCCGACAACCCT
GAACGACCAGGCGTCTTCGTTCATCTATCGGATCGCCACACTCACAACAATG
AGTGGCAGATATAGCCTGGTGGTTCAGGCGGCGCATTTTTATTGCTGTGTTGC
GCTGTAATTCTTCTATTTCTGATGCTGAATCAATGATGTCTGCCATC

Note: The bases mutated are in bold.

The following primers are designed to demonstrate the criticality of last base match on PCR efficiency:

Experiment 1:

CF	CTCACGCTGCTCGTTGAG (highlighted)
CR	TCTGCCACTCATTGTTGTGAGTGTG (highlighted)
WSP1FP	TCATGCATTGCCTGCTCTGCCGG (highlighted)

Where, CF (control forward) and CR (control reverse) are Primers that amplify the 380 bp sequence containing the mutations and is used as PCR positive control.

WSP1FP (wild-type forward) primer is specific for wild type template and ends at G

Two tubes of PCR are set up with following combination of template and primers:

1. Wild template with CF,CR and WSP1FP primer.
2. Mutant template with CF,CR and WSP1FP primer.

For the above two PCR reactions, following conditions with respect to the components are employed-

Reaction Volume 25 µl

Enzyme FasTaq 0.25µl (0.75 Units)

dNTP 1 µl (10 mM mix)

PCR cycle conditions

94 °C	94 °C	55 °C	72 °C	72 °C	4 °C
2 min	5 sec	10 sec	1 sec	10 sec	∞
1 Cycle	← 30 Cycles →			1 Cycle	Hold

Observations [Figure 4 (a)]:

In lane 1, results for amplification of the wild-type template using the designed primers is shown. Both positive control primers (CF and CR) and WSP specific primers have worked, since the primers and template are perfectly complementary to each other.

In lane 2, results for amplification of the mutant template using the designed primers is shown. Only control primers (CF and CR) have worked and the WSP specific primers have failed, as the last base of the template is not complementary to the last base of the designed primer. Hence, no amplification occurs because of last base mismatch.

Experiment 2:

CF	CTCACGCTGCTCGTTGAG
CR	TCTGCCACTCATTGTTGTGAGTGTG
WSP1FP	TCATGCATTGCCTGCTCTGCCGG
WSP1-1FP	TCATGCATTGCCTGCTCTGCCG
WSP1-2FP	TCATGCATTGCCTGCTCTGCC

Where, CF (control forward) and CR (control reverse) are Primers that amplify the 380 bp sequence containing the mutations and is used as PCR positive control, and

Primer WSP1FP is specific to wild type template that ends at ‘G’[N].

Primer WSP1-1FP is a base smaller that ends at ‘G’ [N-1].

Primer WSP1-2FP is 2 bases smaller that ends at ‘C’[N-2].

Two tubes of PCR are set up with following combination of template and primers:

1. Wild template with CF,CR and WSP1FP, WSP1-1FP, WSP1-2FP primer mix.
2. Mutant template with CF,CR and WSP1FP WSP1-1FP, WSP1-2FP primer mix.

5 For the above two PCR tubes, following conditions with respect to the components are employed-

Reaction Volume 25 µl

Enzyme FasTaq 0.25 µl (0.75 Units)

dNTP 1 µl (10 mM mix)

10

PCR cycle conditions

94 °C	94 °C	55 °C	72 °C	72 °C	4 °C
2 min	5 sec	10 sec	1 sec	10 sec	∞
1 Cycle	← 30 Cycles →			1 Cycle	Hold

Observations [Figure 4 (b)]:

15 In lane 1, results for amplification of the wild-type template using the designed primers is shown. Both positive control primers (CF and CR) and WSP specific primers have worked since there are no mutations and thus all the primers bind and amplify the template.

In lane 2, results for amplification of the mutant template using the designed primers is
20 shown. Both positive control primers (CF and CR) and WSP specific primers have worked. Therefore, it is observed that despite mutations in the template sequence, the designed consensus primer set [of Experiment 2] successfully amplifies the mutated template. A comparison of this experiment with experiment 1 above shows that primers having less bases [N-x] than [N] bases of the primer annealing region of the template are
25 still able to successfully amplify even if the mutations are present at the Nth position.

EXAMPLE 3:

In another embodiment, a wild template and a corresponding mutated template is generated in order to showcase the efficacy and success of amplification of the mutated template by the primers designed according to the present disclosure.

Wild Template:

CTCACGCTGCTCGTTGAGTTTTGATTTTGCTGTTTCAAGCTCAACACGCAGTTT
CCCTACTGTTAGCGCAATATCCTCGTTCTCCTGGTCGCGGCGTTTGATGTATT
GCTGGTTTCTTTCCCGTTCATCCAGCAGTTCCAGCACAATCGATGGTGTTACC
AATTCATGGAAAAGGTCTGCGTCAAATCCCCAGTCGTCATGCATTGCCTGCTC
TGCCGGTTCACGCTGTGCCTGAGAGTTAATTTCGCTCACTTCGAACCTCTCTG
TTTACTGATAAGTTCCAGATCCTCCTGGCAACTTGCACAAGTCCGACAACCCT
GAACGACCAGGCGTCTTCGTTCATCTATCGGATCGCCACACTCACAACAATG
AGTGGCAGATATAGCCTGGTGGTTCAGGCGGCGCATTTTTATTGCTGTGTTGC
GCTGTAATTCTTCTATTTCTGATGCTGAATCAATGATGTCTGCCATC

Mutant Template:

CTCACGCTGCTCGTTGAGTTTTGATTTTGCTGTTTCAAGCTCAACACGCAGTTT
CCCTACTGTTAGCGCAATATCCTCGTTCTCCTGGTCGCGGCGTTTGATGTATT
GCTGGTTTCTTTCCCGTTCATCCAGCAGTTCCAGCACAATCGATGGTGTTACC
AATTCATGGAAAAGGTCTGCGTCAAATCCCCAGTCGTCATGCATTGCCTGCTC
TGCCGCTTCACGCAGTGCCTGAGAGTTAATTTCGCTCACTTCGAACCTCTCTG
TTTACTGATAAGTTCCAGATCCTCCTGGCAACTTGCACAAGTCCGACAACCCT
GAACGACCAGGCGTCTTCGTTCATCTATCGGATCGCCACACTCACAACAATG
AGTGGCAGATATAGCCTGGTGGTTCAGGCGGCGCATTTTTATTGCTGTGTTGC
GCTGTAATTCTTCTATTTCTGATGCTGAATCAATGATGTCTGCCATC

The following primers are designed to demonstrate the criticality of last base match on PCR efficiency and how the present primer designing protocol overcomes it:

Experiment 1:

CRF	TCTGCCACTCATTGTTGTGAGTGTG (highlighted)
WSP1FP	TCATGCATTGCCTGCTCTGCCGG (highlighted)

Where, CRF (Control Reverse Fluorescent) primer is labeled at 5'- end with a fluorescent tag FAM (carboxy fluorescein) and,

WSP1FP (wild-type forward) primer is specific to wild type template (ends at G)

Two tubes of PCR are set up with following combination of template and primers:

1. Wild template with CRF and WSP1FP primer.
2. Mutant template with CRF and WSP1FP primer.

For the above two PCR tubes, following conditions with respect to the components are employed-

Reaction Volume 25 µl

Enzyme FasTaq 0.25 µl (0.75 Units)

dNTP 1 µl (10 mM mix)

PCR cycle conditions

94 °C	94 °C	55 °C	72 °C	72 °C	4 °C
2 min	5 sec	10 sec	1 sec	10 sec	∞
1 Cycle	← 30 Cycles →			1 Cycle	Hold

After PCR, 1 micro litre of PCR product is taken in a vial and mixed with 8 micro litre of Formamide (Hi-DiTM Applied Biosystems, Part No. 4311320) and 1 micro lit of Size Standard (Applied Biosystems, Part No.4322682) GeneScan™ -500 LIZ®. The mix is denatured 94 ºC for 10 min and quick-chilled on ice.

The quick chilled samples are loaded on the Genetic Analyzer (Applied Biosystems® 3500XL) and the samples are run and analyzed as per manufacturer's protocol.

Observations [Figure 5 (a)]:

In Lane-A, results for amplification of the wild-type template using the designed primers is shown. Peak seen at 184 bp indicates successful PCR. Hence, both positive control primer and WSP specific primer have worked since there are no mutations and thus all the primers bind and amplify the template.

In Lane-B, results for amplification of the mutant template using the designed primers is shown. No peak is seen indicating failure of PCR due to last base mismatch in the mutant template. The WSP specific primers have failed, as the last base is not complementary to the last base of the designed primer. Hence, no amplification occurs because of last base mismatch.

Experiment 2:

CRF	TCTGCCACTCATTGTTGTGAGTGTG
WSP1FP	TCATGCATTGCCTGCTCTGCCGG
WSP1-1FP	TCATGCATTGCCTGCTCTGCCG
WSP1-2FP	TCATGCATTGCCTGCTCTGCC

Where, CRF (Control Reverse Fluorescent) primer is labeled at 5'- end with a fluorescent tag FAM (carboxy fluorescein) and,

WSP1FP (wild-type forward) primer is specific to wild type template (ends at G),

Primer WSP1-1FP is a base smaller with respect to WSP1FP ends at ‘G’,
Primer WSP1-2FP is 2 bases smaller with respect to WSP1FP ends at ‘C’.

Two tubes of PCR are set up with following combination of template and primers:

1. Wild template with CRF and WSP1FP primer.
2. Mutant template with CRF and WSP1FP primer mix.

For the above two PCR tubes, following conditions with respect to the components are employed-
Reaction Volume 25 µl
Enzyme FasTaq 0.25 µl (0.75 Units)
dNTP 1 µl (10 mM mix)

PCR cycle conditions

94 °C	94 °C	55 °C	72 °C	72 °C	4 °C
2 min	5 sec	10 sec	1 sec	10 sec	∞
1 Cycle	← 30 Cycles →			1 Cycle	Hold

After PCR, 1 micro litre of PCR product is taken in a vial and mixed with 8 micro litre of Formamide (Hi-DiTM Applied Biosystems, Part No. 4311320) and 1 micro lit of Size Standard (Applied Biosystems, Part No.4322682) GeneScan™ -500 LIZ®. The mix is denatured 94 °C for 10 min and quick-chilled on ice.
The quick chilled samples are loaded on the Genetic Analyzer (Applied Biosystems® 3500XL) and the samples are electrophoresed and analyzed as per manufacturer’s protocol.

Observations [Figure 5 (b)]:

In Lane-C, results for amplification of the wild-type template using the designed primers is shown. Peak is seen at 184 bp indicating successful PCR. Both positive control primer

and WSP specific primers have worked since there are no mutations and thus all the primers bind and amplify the template. .

In Lane-D, results for amplification of the mutant template using the designed primers is shown. Peak is seen at 184 bp indicating successful PCR due to employment of novel primer set. Both positive control primer and WSP specific primers have worked. Therefore, it is observed that despite mutations in the template sequence, the designed consensus primer set [of Experiment 2] successfully amplifies the mutated template. A comparison of this experiment with experiment 1 above shows that primers having less bases [N-x] than [N] bases of the primer annealing region of the template are still able to successfully amplify even if the mutations are present at the Nth position.

EXAMPLE 4:

In regular PCRs, there are 2 primers: one forward primer and one reverse primer. The forward and reverse primers anneal on the template on the lower strand and upper strand respectively and amplify the region of the template between the annealing points.

Conventionally, Multiplex PCR protocols uses multiple sets/ pairs of primers that specifically and independently anneal at different regions on the template(s) and amplify multiple amplicons from the template(s). Also, each set of primer anneals specifically at the respective target template region only.

In the present disclosure, multiple sets of primers that specifically and independently anneal at the same region on the template and amplify the same amplicon from the template are used. Also, all or more than one primer sets can anneal the target template region and amplify the same.

EXAMPLE 5:

STEP-WISE PCR-BASED PROTOCOL

In an embodiment the step-wise PCR based protocol is for molecular diagnostic applications and the steps followed are provided below:

1. Step-1: Designing of primers.
2. Step-2: Isolation of sample target DNA.
3. Step-3: Performing PCR using standardized PCR cycle conditions using the primers, amplification reagents and sample target DNA.
4. Step-4: Detection of PCR product is carried by loading the product on Agarose gels.

Finally, if PCR worked, it would indicate presence of the contamination/ infection. Failure of PCR suggests no contamination/ infection.

The most critical step within the 4 steps mentioned above is the designing of the primers (Step-1). The primer sequences have to be chosen such that there are no mismatches between the template sequence and the primer sequence, particularly at the 3'-end of the primer. In an exemplary embodiment, a primer set comprising a plurality of primers is designed based on the nucleotide sequence of the template and the possible mutated bases on the template as reported in the literature.

Any one or more mismatches within the last 3 bases in the 3'-end would result into sub-optimal or failure PCRs. Thus, several sets of primers are usually designed for a particular organism/target and the performance of the primers is validated for all strains/variants available in the geographical region.

EXAMPLE 6:

This example provides for a comparative analysis between the present disclosure and prior art; depicting the absence of amplification when mutations are present in the template in prior art studies and on the other hand irrespective of the presence of mutations the amplification of the template in the instant disclosure [Figure 2].

EXAMPLE 7:

The size of an amplicon is the distance / size between the bases between the 5'-ends of the forward and reverse primers used for PCR. Figure-3 shows Amplicon from conventional PCR and PCR based on the present disclosure.

The PCR amplicon size is always the same as the 5'-ends of both the primers (forward as well as reverse) end at identical points.

5 The current disclosure has applications in cancer detection as well as detection of genetic disorders apart from its applications to detect microbes. Some of the applications are captured below.

1. Multiple Drug Resistance (MDR) Tuberculosis, Drug-resistant Malaria,
10 Methicilin Resistant Staphylococcus aureus (MRSA), etc, occur when mutations are introduced within genes responsible for drug resistance.
2. Thus, if primers are designed for amplifying the hotspots (regions where most mutations occur) of the organism, it would serve 2 purposes; detection of the microbe as well as presence of mutations / MDR (detected by sequencing the
15 amplicon).
3. Amplification of hotspots is possible through the current disclosure as it would allow PCR amplifications as NOT all 3'-end positions of all primers would have mutations in the primer annealing regions.

20 **Advantages:**

a. Molecular diagnostic kits with primers designed as per the present disclosure would make the protocols fail-proof. At present, a PCR-based kit with a sensitivity of 90% is regarded as an "excellent" kit.
b. Over-all success rate (in terms of sensitivity and specificity) for molecular
25 diagnostics will improve resulting in better acceptance of the molecular protocols.
c. Molecular diagnostic kit development programs will benefit as large scale validation process will not be required.

We Claim:

1. A primer set comprising plurality of primers having a maximum length N, ranging from about 19 to about 45 nucleotides, wherein each primer has a length selected from a group comprising N, N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, N-11, N-12, N-13, N-14, N-15, N-16, N-17, N-18, N-19, N-20, N-21, N-22, N-23, N-24, N-25 and N-26 or any combination thereof, wherein the primers are corresponding to a consensus sequence flanking a region of a target nucleotide sequence to be amplified, said region having mutation or suspected of having mutation at N^{th} position.
2. The primer set as claimed in claim 1, wherein one of the primers in the set is having a length N and remaining primers in the set are having a length ranging from about N-1 to about N-26 in any order thereof.
3. A kit for amplifying a region of target nucleotide sequence having mutation or suspected of having mutation at N^{th} position, said kit having a primer set comprising plurality of primers having a maximum length N, ranging from about 19 to about 45 nucleotides, wherein each primer has a length selected from a group comprising N, N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, N-11, N-12, N-13, N-14, N-15, N-16, N-17, N-18, N-19, N-20, N-21, N-22, N-23, N-24, N-25 and N-26 or any combination thereof, wherein the primers are corresponding to a consensus sequence flanking said region, optionally along with DNA polymerase and nucleic acid amplification reagent selected from a group comprising magnesium chloride, dNTPs and buffer or any combination of reagents thereof.
4. The kit as claimed in claim 3, wherein the kit is a reaction mixture used for amplifying said region of the target nucleotide sequence.
5. The primer set as claimed in claim 1 and the kit as claimed in claim 3, wherein the amplification is carried out by technique selected from a group comprising Conventional Polymerase Chain Reaction, HotStart Polymerase Chain Reaction, Real Time Polymerase Chain Reaction and Isothermal Amplifications.
6. The primer set as claimed in claim 1 and the kit as claimed in claim 3, wherein the mutation is selected from a group comprising unpredicted sequence variations,

SNPs, deletions, alternate splice products, microsatellites and gene rearrangements or any combination thereof.

7. A method of obtaining a primer set comprising plurality of primers having a maximum length N, ranging from about 19 to about 45 nucleotides, wherein each primer has a length selected from a group comprising N, N-1, N-2, N-3, N-4, N-5, N-6, N-7, N-8, N-9, N-10, N-11, N-12, N-13, N-14, N-15, N-16, N-17, N-18, N-19, N-20, N-21, N-22, N-23, N-24, N-25 and N-26 or any combination thereof, said method comprising acts of:
    a. identifying a region for amplification having mutation or suspected of having mutation at N^{th} position in a target nucleotide sequence, followed by observing a consensus sequence flanking the said N^{th} position; and
    b. synthesizing plurality of primers corresponding to the consensus sequence, wherein length of the primers range from about 19 to about 45 nucleotides to obtain said primer set.
8. A method of amplifying a region of target nucleotide sequence having mutation or suspected of having mutation at N^{th} position, said method comprising act of subjecting the target nucleotide sequence to the reaction mixture as claimed in claim 4, for amplifying the said region.
9. The method as claimed in claim 8, wherein the amplification with said reaction mixture provides amplified product of the mutated region.
10. The method as claimed in claim 9, wherein the amplified product is detected by techniques selected from a group comprising Agarose based detection, spectrophotometric detection and fluorometric detection or any combination thereof.
11. The method as claimed in claim 8, wherein primers of the reaction mixture amplifies said region of the target nucleotide sequence by binding to a consensus sequence flanking the region having mutation or suspected of having mutation at N^{th} position.
12. The method as claimed in claim 8, wherein primers of the reaction mixture anneal at same location at 3' end of the consensus sequence and at different locations at 5' end of the consensus sequence.

13. The method as claimed in claim 8, wherein the amplification is carried out by technique selected from a group comprising Conventional Polymerase Chain Reaction, HotStart Polymerase Chain Reaction, Real Time Polymerase Chain Reaction and Isothermal Amplifications.
14. The methods as claimed in claims 7 and 8, wherein the mutation is selected from a group comprising unpredicted sequence variations, SNPs, deletions, alternate splice products, microsatellites and gene rearrangements or any combination thereof.
15. The primer set as claimed in claim 1, the kit as claimed in claim 3 and the methods as claimed in claims 7 and 8, wherein the target nucleotide sequence is selected from a group comprising prokaryotic and eukaryotic genomic sequence or a combination thereof.

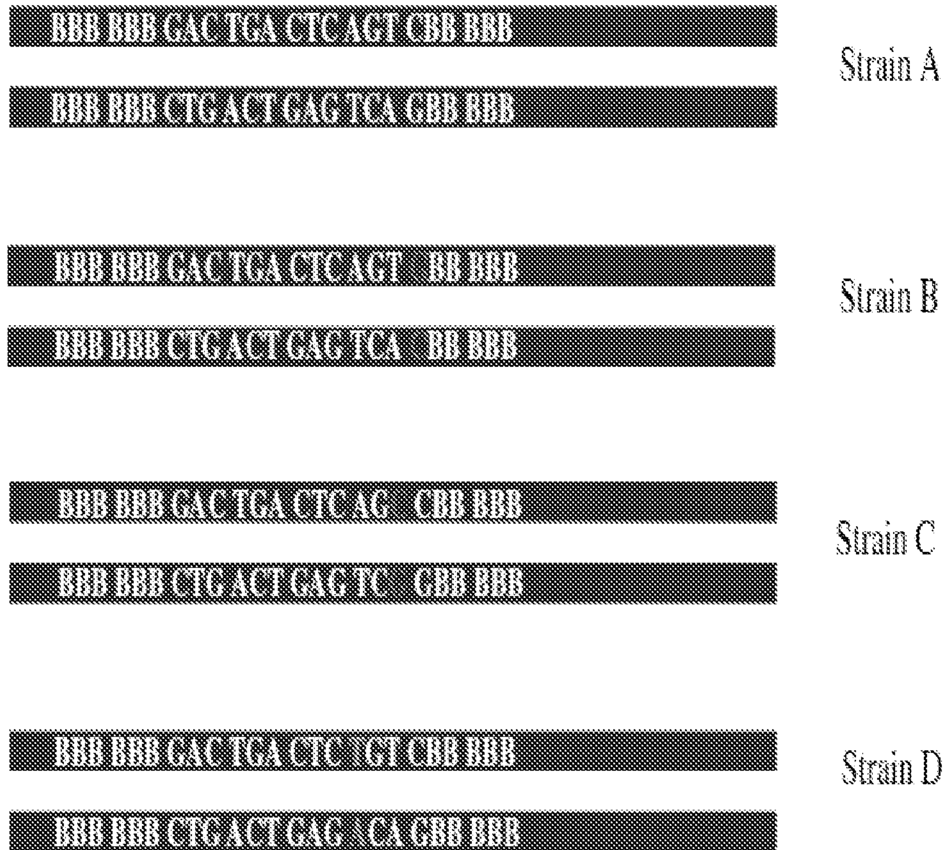

FIGURE 1

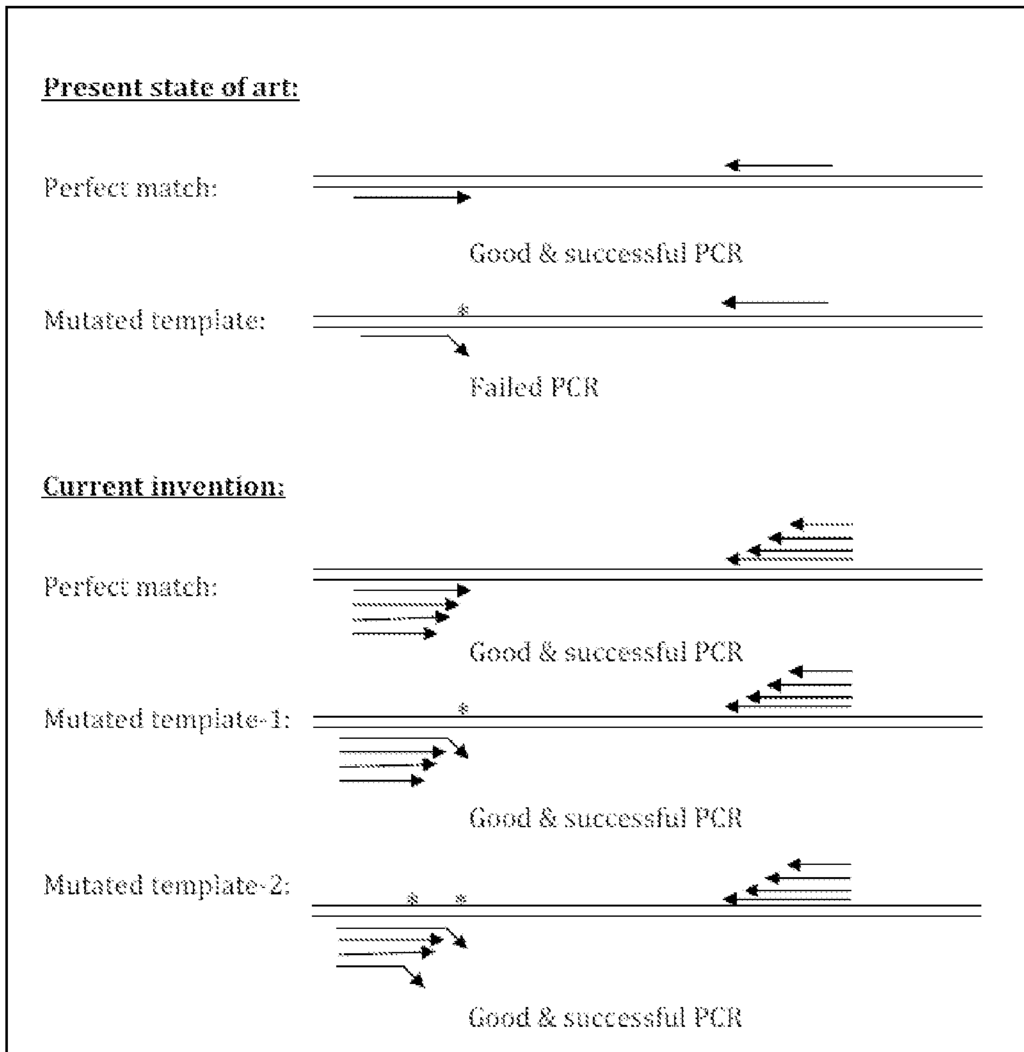

FIGURE 2

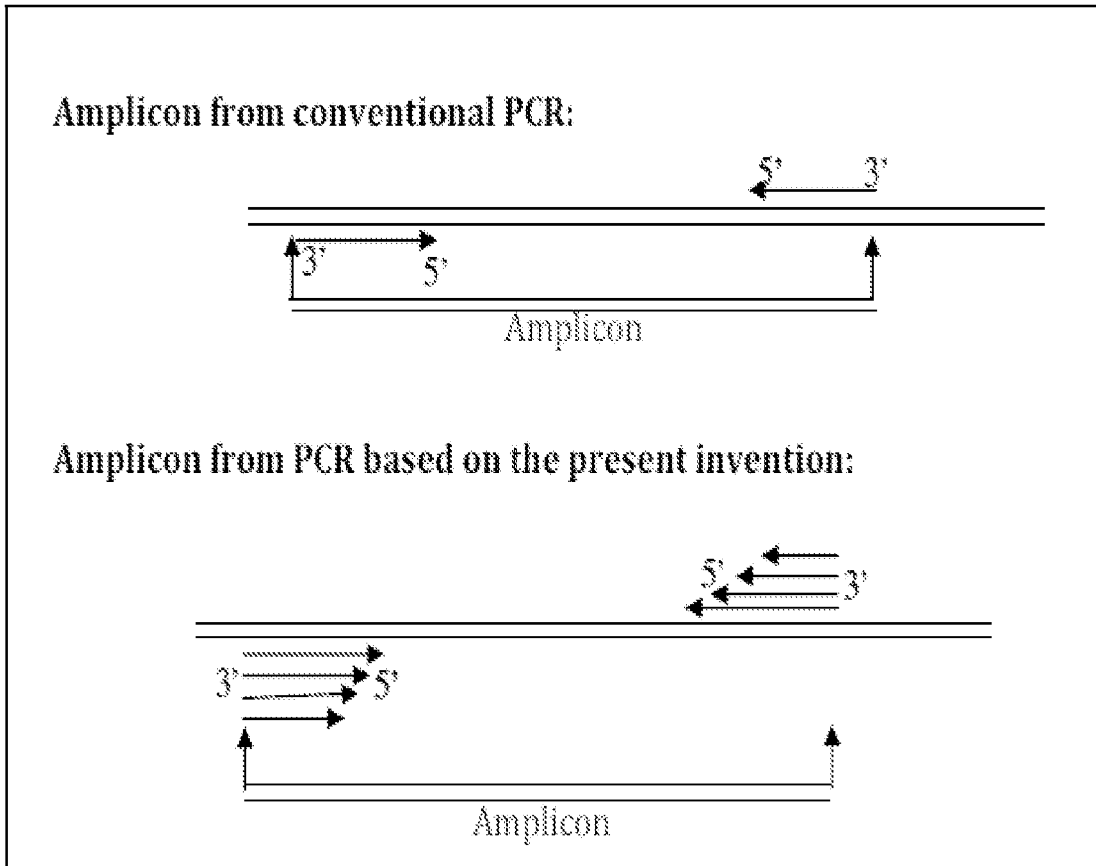

FIGURE 3

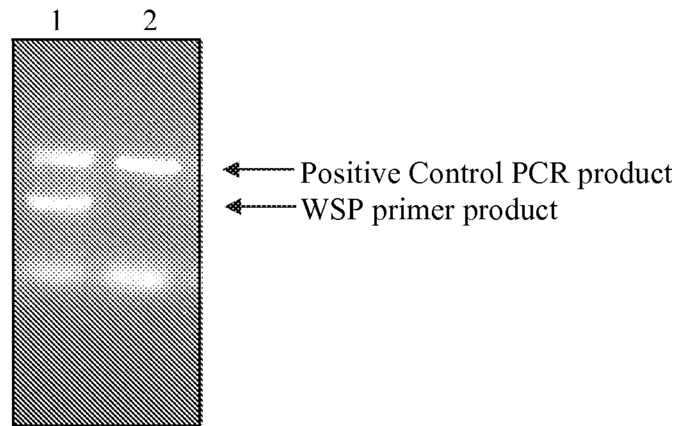

FIGURE 4 (a)

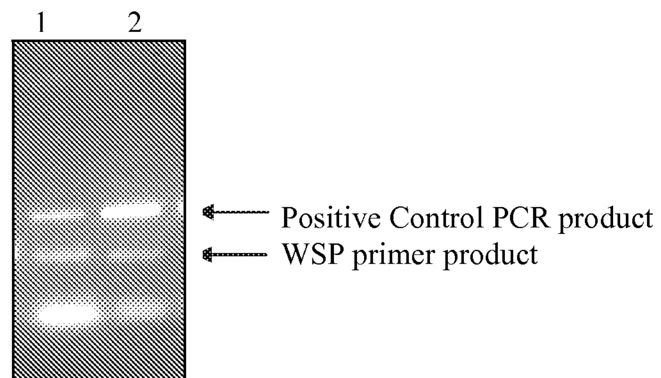

FIGURE 4 (b)

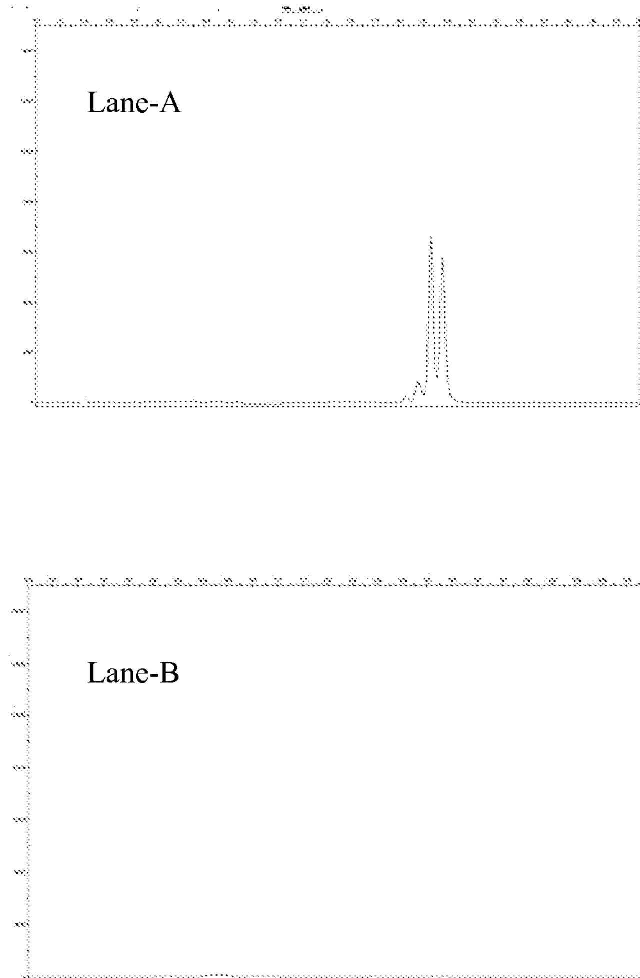

FIGURE 5 (a)

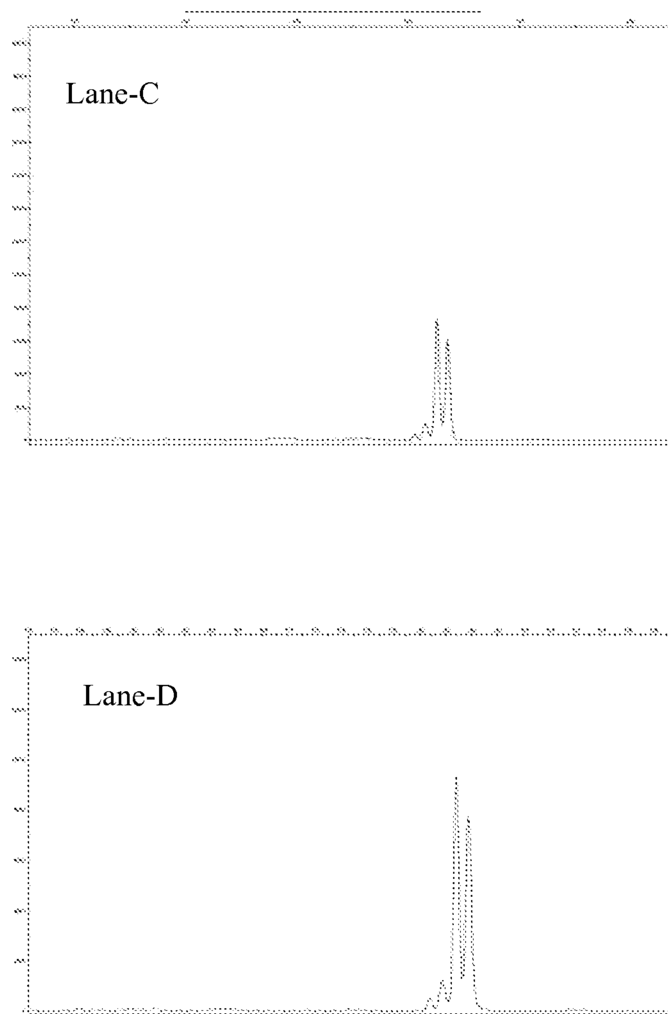

FIGURE 5 (b)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2012/051517

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68 (OCT 2005)

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)

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)

WPI, EPODOC, MEDLINE, HCAPLUS, BIOSIS, BIOTECHABS & keywords: primer, stair primer, set, kit, amplify, varying length, same target, mutant sequence, 3 prime mutation, shared 5 prime, and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C   [X] 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	Date of mailing of the international search report
25 June 2012	26 June 2012

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INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/IB2012/051517
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 1995/031568 A2 (UNIVERSITE DE RENNES I) 23 November 1995 See Abstract; pages 1-9; page 15, lines 31-36; Figures 1, 2	1-15
X	COLIMON, R. et al., "New types of primers (stair primers) for PCR amplification of the variable V3 region of the human immunodeficiency virus", Journal of Virological Methods, 1996, vol. 58, pages 7-19 See Abstract; Materials and Methods; Figures 1, 2	1-15
X	MINJOLLE, S. et al., "Amplification of the Six Major Human Herpesviruses from Cerebrospinal Fluid by a Single PCR", Journal of Clinical Microbiology, 1999, vol. 37, No. 4, pages 950-953 See Abstract; Materials and Methods	1-15
X	BOUQUILLON, C. et al., "Simultaneous Detection of 6 Human Herpesviruses in Cerebrospinal Fluid and Aqueous Fluid by a Single PCR Using Stair Primers", Journal of Medical Virology, 2000, vol. 62, pages 349-353 See Abstract; Materials and Methods	1-15

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/IB2012/051517**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 1995/031568 A2	23 Nov 1995	EP 0720660 A1	10 Jul 1996
		EP 0720660 B1	13 Feb 2002
		FR 2720077 A1	24 Nov 1995
		FR 2720077 B1	09 Aug 1996
		WO 9531568 A2	23 Nov 1995

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.