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(71) Applicant: OMIX RESEARCH AND DIAGNOSTIC LABORATORIES PRIVATE LIMITED [IN/IN]; No 15, Chaithanya Armadale, Whitefield Main Road, Varthur Kodi, Karnataka, Bangalore 560066 (IN).

(72) Inventor: ADAK, Sudeshna; Villa 15, Chaithanya Armadale, Whitefield Main Road, Karnataka, Bangalore 560066 (IN).

(74) Agent: R. Sridhar et al.; K&S Partners | Intellectual Property Attorneys, 4121/B, 6th Cross, 19A Main, HAL II Stage (Extension), Bangalore 560038 (IN).

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(54) Title: A METHOD, A KIT AND APPLICATION THEREOF

(57) Abstract: The present disclosure describes a method for detecting nucleic acids in a sample, said method comprises combination of primary amplification and secondary amplification. The disclosure further describes a kit comprising microplate and buffer or water or both. The said method and the kit of the present disclosure detects nucleic acids in a sample without any non-specific amplification, thereby not leading to any false positives. The said method and the kit of the present disclosure is very sensitive and accurate in detecting nucleic acids in a sample.



“A METHOD, A KIT AND APPLICATION THEREOF”**TECHNICAL FIELD**

The present disclosure describes a method for detecting nucleic acids, said method comprises combination of primary amplification and secondary amplification, wherein the primary amplification is nucleic acid amplification and secondary amplification is signal amplification. The disclosure further describes a Kit comprising microplate comprising primers, enzymes, reagents, probes, buffering reagents and nucleoside triphosphate, buffer or water including but not limited to nuclease free water and double distilled water, or both.

BACKGROUND OF THE DISCLOSURE

Variety of techniques are known to amplify nucleic acids such as polymerase chain reaction, ligase chain reaction, loop-mediated isothermal amplification (LAMP), nucleic acid sequence based amplification, simple amplification based assay, helicase dependent amplification and rolling circle amplification. However, it is observed that well known nucleic acid amplification techniques cause non-specific amplification of the nucleic acid, thereby hampering the detection of nucleic acid, causing low specificity during detection of nucleic acid in a sample.

Further loop-mediated isothermal amplification (LAMP) has been well established as a powerful isothermal amplification, able to amplify low copy number nucleic acids. However, those well versed with LAMP also are aware of the challenges of deploying LAMP in the field due to its propensity for non-specific amplification leading to false positives. While LAMP works very well in controlled laboratory settings, even small variations that occur in the protocol or the reagents under storage can lead to non-specific amplification in negative controls or failure in the LAMP reactions.

In view of the above said, there are limitations in effectively detecting nucleic acid from a sample and there is a need to overcome such limitations. The description of the instant specification intends to address such limitations.

SUMMARY OF THE DISCLOSURE

The present disclosure describes a method for detecting nucleic acid, said method comprises combination of primary amplification and secondary amplification, wherein the primary amplification is nucleic acid amplification and the secondary amplification is signal amplification.

The present disclosure further describes a kit comprising microplate having wells comprising primers, probes, enzymes, reagent and nucleoside triphosphate, and buffer or water or both.

BRIEF DESCRIPTION OF THE 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 figures together with detailed description below, are 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 illustrates a likely design of the microplate of the instant disclosure.

FIGURE 2 illustrates the outcome of the combination of primary amplification and secondary amplification of the present disclosure, wherein **FIGURE 2a** illustrates output of the primary amplification using negative control and 10copies/ μ l and 100 copies/ μ l of pathogen DNA and **FIGURE 2b** illustrates the fold change overtime in the secondary amplification.

FIGURE 3 illustrates specificity of the method of the present disclosure, wherein **FIGURE 3a** illustrates output of the induced non-specific amplification in 4 of 8 negative controls during primary amplification, and **FIGURE 3b** illustrates the output of the secondary amplification, wherein the all 8 negative controls showed only basal signal, demonstrating absence of non-specific amplification detection.

FIGURE 4 illustrates the output of the secondary amplification of the present disclosure, detecting CTX-M-15 in about 10 copies/ μ l of DNA in a sample.

FIGURE 5 illustrates detection of *Plasmodium falciparum* in crude blood lysate.

FIGURE 6 illustrates molecular beacon probe and its mechanism of action during enzyme assisted target recycling.

FIGURE 7 illustrates the comparison of the polymerase chain reaction and the method of the present disclosure.

FIGURE 8 illustrates the output of the kit with DNA extracted from clinical samples of *Plasmodium falciparum*. DNA from 10 samples that were confirmed by microscopy and rapid

diagnostics were used. All 10 samples showed positive amplification, above a threshold of 40, while the negative controls used did not show any amplification.

DETAILED DESCRIPTION

5 The present disclosure describes a method for detecting nucleic acid, said method comprises combination of primary amplification and secondary amplification, wherein the primary amplification is nucleic acid amplification and the secondary amplification is signal amplification.

10 In an embodiment, the method detects target nucleic acid, wherein the method combines primary amplification and secondary amplification.

In an embodiment, the nucleic acid includes but is not limited to bacterial DNA, bacterial RNA, viral DNA, viral RNA and fungal DNA found in water, food, animal and human.

15 In an embodiment, the method of present disclosure is a synergistic combination of nucleic acid amplification and signal amplification, which does not cause non-specific amplification of the nucleic acid, thereby aids in detecting as low as 1 copy/ μ l of DNA from the sample.

20 In an embodiment, the method of present disclosure does not lead to false positives during detection of the nucleic acid.

In an embodiment, the primary amplification described in the method of the present disclosure is target nucleic acid amplification, wherein the primary amplification includes but is not
25 limited to polymerase chain reaction, ligase chain reaction, loop-mediated isothermal amplification, nucleic acid sequence based amplification, simple amplification based assay, helicase dependent amplification and rolling circle amplification.

In an exemplary embodiment, the primary amplification described in the method of the present
30 disclosure is loop mediated isothermal amplification (LAMP).

In an embodiment, the secondary amplification described in the method of the present disclosure includes but is not limited to enzyme assisted target recycling (EATR).

35 In an exemplary embodiment, the method for detecting nucleic acid comprises combination of loop mediated isothermal amplification and enzyme assisted target recycling.

In another embodiment, the method for detecting target nucleic acid comprises combination of loop mediated isothermal amplification and enzyme assisted target recycling.

5 In an embodiment, the primary amplification including but not limited to loop mediated isothermal amplification produces copies of target nucleic acid by amplification from a sample in a reaction mixture comprising oligonucleotide primers, enzymes, buffers, nucleoside triphosphate and any other component required for optimal primary amplification.

10 In an alternate embodiment, the primary amplification including but not limited to loop mediated isothermal amplification produces copies of target nucleic acid by amplification from a sample in a reaction mixture comprising oligonucleotide primers, enzymes, buffers, nucleoside triphosphate and any other component required for optimal primary amplification and oligonucleotide probes (EATR probes).

15 In a non-limiting embodiment, the enzymes employed during the primary amplification includes but is not limited to Taq polymerases, Bst polymerase-Bst 2.0, Bst polymerase-Bst 3.0, Bst large fragment polymerase, GspSSD LF DNA polymerase (from Optiigene) and OmniAmp (from Lucigen).

20 In another embodiment, the buffers employed during the primary amplification includes but is not limited to mixtures containing some salts (chlorides, sulphates or acetates) of magnesium, potassium, or sodium, Tris HCL; DTT, BSA and volume displacement reagents such as PEG.

25 In another non-limiting embodiment, the reagent employed during the primary amplification includes but is not limited to dNTPs in a solution or buffer.

In an embodiment, the primary implication is performed at temperature ranging from about 25°C to 70°C.

30 In an embodiment, the enzymes employed during the loop mediated isothermal amplification includes but is not limited to Bst polymerase-Bst 2.0, Bst polymerase-Bst 3.0, Bst large fragment polymerase, GspSSD LF DNA polymerase (from Optiigene) and OmniAmp (from Lucigen).

35 In an embodiment, the buffers employed during loop mediated isothermal amplification incudes but is not limited to salts comprising Mg salts comprising Mb and Tris, potassium salts.

In an embodiment, the reagent employed during loop mediated isothermal amplification includes but is not limited to dNTPs, or a combination of dATP, DUTP, dGTP, dCTP, and Uracil DNA glycosylase.

- 5 In another embodiment, the secondary amplification including but not limited to enzyme assisted target recycling comprises at least one labelled oligonucleotide probe, buffers, reagents and any other component required for optimal secondary amplification. The enzyme employed during secondary amplification is required to have 3' to 5' exonuclease activity in cleaving double stranded DNA from 3' to 5' direction. And, such enzyme includes but is not limited to exonuclease III.

10

In a non-limiting embodiment, the oligonucleotide probe employed during the secondary amplification includes but is not limited to molecular beacon probe, strand displacement probes, hybridization probe or any probe that can be used in enzyme assisted target recycling.

- 15 In an exemplary embodiment, in the method of the present disclosure, amplicons from the primary amplification including but not limited to loop mediated isothermal amplification (LAMP) are hybridized to molecular beacon probe during secondary amplification including but not limited to enzyme assisted target recycling (EATR), wherein the molecular probe beacon ensure that only specific amplicon from the LAMP are hybridized leading to signal
20 amplification in the secondary amplification.

- In an embodiment, EATR is amenable to multiplexing and overcomes any challenges that is associated with multiplexing with LAMP in the method of the present disclosure, thereby detection of the minimum DNA or low copy number nucleic acid, as low as 1 copy/ μ l DNA in
25 the sample. And, the method of the present disclosure is accurate without any non-specific nucleic acid amplification and detection.

- In an embodiment, the primers for the primary amplification are designed in such a way that they always work in conjunction with the oligonucleotide probes of the secondary
30 amplification

- In an embodiment, the amplicons of primary amplification, in case of LAMP, are single stranded nucleic acid with several inverted repeats of target nucleic acid and cauliflower like structure formed by annealing between alternatively inverted repeats of the target nucleic acid.

35

In an embodiment, the method of detecting the nucleic acid comprises steps of:

contacting sample comprising nucleic acid with the nucleic acid amplification reaction mixture, followed by amplifying the nucleic acid to obtain amplicon;
hybridizing the amplicon and oligonucleotide probe; and
contacting the hybridized amplicon and the probe with enzyme and cleaving the probe
5 by the enzyme to release the amplicon, wherein the cleaved probe produces signal.

In another embodiment, the method of detecting the nucleic acid comprises steps of:

contacting sample comprising nucleic acid with the nucleic acid amplification reaction mixture, followed by amplifying the nucleic acid to obtain amplicon;
10 hybridizing the amplicon and oligonucleotide probe;
contacting the hybridized amplicon and the probe with enzyme and cleaving the probe by the enzyme to release the amplicon, wherein the cleaved probe produces signal; and
hybridizing the released amplicon with another oligonucleotide probe, followed by
cleaving the probe by the enzyme to release the amplicon, wherein the cleaved probe
15 produces signal to detect the nucleic acid.

In an embodiment, the step of hybridizing the amplicon and oligonucleotide probe is repeated plurality of times so that the enzyme can cleave the probes to amplify the signal to detect the nucleic acid.

In an embodiment, the step of hybridizing the amplicon and oligonucleotide probe is repeated at least one time, at least 2 times, at least 3 times, at least 4 times, at least 5 times, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times so that the enzyme can cleave the probes to amplify the signal to detect the nucleic acid.

In an embodiment, the during the method of hybridizing the probe to amplicon, cleaving the probe of the hybridized probe and amplicon produces amplification of the signal to will aid in detecting the nucleic acid as of 1copy/ μ l of DNA in the sample.

In an embodiment, the during the method of hybridizing the probe to amplicon, cleaving the probe of the hybridized probe and amplicon produces amplification of the signal to will aid in detecting the nucleic acid as of 5 copies/ μ l of DNA.

In an embodiment, the during the method of hybridizing the probe to amplicon, cleaving the probe of the hybridized probe and amplicon produces amplification of the signal to will aid in detecting the nucleic acid as of 10 copies/ μ l of DNA.

In an embodiment, the nucleic acid amplification reaction mixture comprises-

- i. Primer selected from a group comprising outer primer, inner primer, middle primer and loop primer, or any combination thereof;
- 5 ii. Enzyme selected from a group comprising Taq polymerase, Bst polymerase and other DNA polymerase that supports amplification of nucleic acid;
- iii. Buffer selected from a group comprising magnesium chloride, magnesium sulphate, magnesium acetate, potassium chloride, potassium sulphate, potassium acetate, sodium chloride, sodium sulphate, sodium acetate, Tris HCL and DTT; and
- 10 iv. Reagent selected from a group comprising Poly ethylene glycol, solution comprising dNTPs;

In an embodiment, the primers in the nucleic acid amplification reaction mixture are outer primer, inner primer, middle primer and loop primer.

15

In an embodiment, the inner primer is Pf-FIP and Pf-BIP, set forth as SEQ ID No. 1 and SEQ ID No. 2, respectively and the outer primer is Pf-F3 and Pf-B3, set forth as SEQ ID No. 7 and SEQ ID No. 8, respectively,

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In another embodiment, the inner primer is CTXM-FIP and CTXM, set forth as SEQ ID No. 3 and SEQ ID No. 4, respectively, and the outer primer is CTXM-F3 and CTXM-B3, set forth as SEQ ID No. 9 and SEQ ID No. 10, respectively

25

In an embodiment, the inner primer is 16S-FIP and 16S-BIP, set forth as SEQ ID No. 5 and SEQ ID No. 6, respectively, the outer primer is 16S-F3 and 16S-B3, set forth as SEQ ID No. 11 and SEQ ID No. 12, respectively and the loop primer is 16S-LF1 and 16S-LB1, set forth as SEQ ID No. 13 and SEQ ID No. 14, respectively

SEQ ID	Description	Sequence
SEQ ID No. 1	Inner primer-PF-FIP	TGACTACCCGTCTGTTATGAACACTCTTCA ATATGCTTTTATTGCTTTTG
SEQ ID No. 2	Inner primer-PF-BIP	ACTACAGCATGGAATAACAAATATGAATA AGCCTCCTATTAATCGTAACTAAGCTAATA AG
SEQ ID No. 3	Inner primer-CTXM-FIP	GGCCGCGCTAAGCTCAGTTAACTATAATCC GATTGCGGAAA
SEQ ID No. 4	Inner primer-CTXM-BIP	CGACAGCTGGGAGACGAAACGAATGGCGG TGTTTAACG

SEQ ID No. 5	Inner primer-16s-FIP	CTTTCGCACCTGAGCGTCAGTCTGCGGTGA AATGCGTAGAGAT
SEQ ID No. 6	Inner primer-16s-BIP	TTGGAGGTTGTGCCCTTGAGCGAATTAAAC CACATGCTCCAC
SEQ ID No. 7	Outer primer-PF-F3	TTTGCTTTGTTCAAAATAAGG
SEQ ID No. 8	Outer primer-PF-B3	GTAGTCCGTCTCCAGAAAATC
SEQ ID No. 9	Outer primer-CTXM-F3	GTGAAAGCGAACCGAATCTG
SEQ ID No. 10	Outer primer-CTXM-B3	GTCAGATTCCGCAGAGTTTG
SEQ ID No. 11	Outer primer-16s-F3	GGAATTACTGGGCGTAAAGC
SEQ ID No. 12	Outer primer-16s-B3	TTTCAACAACACGAGCTGACG
SEQ ID No. 13	Loop primer_LF1	CCTTCGCCACCGGTATTC
SEQ ID No. 14	Loop primer_LB1	GTACGGCCGCAAGGTAA
SEQ ID No. 15	pf probe	AACTCAAAGTCATGATTGAGTTCATTGTG
SEQ ID No. 16	CTXM probe	CATCGCC GTACAGCGATAACGTGGCGATG
SEQ ID No.17	16s probe	ACGTCATAGGTGGGGATGACGTCAAGTCA
SEQ ID No.18	16s probe_1	ATGACTTATGACGTCAAGTCATCATGGCC
SEQ ID No.19	16s probe_2	ATCCCCAGAGGAAGGTGGGGATGACGTCA
SEQ ID No. 20	16s probe_3	ACTTGACGGGATGACGTCAAGTCATCATG
SEQ ID No. 21	16s probe_4	ATGATGAACGTCAAGTCATCATGGCCCTT

In an embodiment, the outer primer, middle primer and loop primer has melting temperature of about 55°C to 61°C, respectively.

- 5 In an embodiment, the inner primer has melting temperature of about 62°C to 66°C.

In an embodiment, the GC content of the outer primer, middle primer, loop primer and inner primer is about 40% to 60%.

- 10 In an embodiment, the amplification of the nucleic acid is carried for a period of about 30 minutes to 90 minutes at temperature ranging from about 60°C to 70°C, and for a period of about 5 minutes to 10 minutes at temperature ranging from about 25°C to 40°C.

- 15 In an embodiment, during amplification of the nucleic acid the amplicon is about 300bp to 400bp. To ensure that the LAMP amplicon length is in the said range of about 300bp to 400bp, the following strategy is followed. Upon designing the oligonucleotide probe, LAMP forward primers are designed in the flanking region within about 1bp to 250bp upstream and LAMP backward primers are designed in about 1bp to 250bp downstream (ideally should not exceed 150bp). The primers thus designed will produce primary amplicons that provide a template to
20 which the probe will bind.

In an embodiment, the oligonucleotide probe includes but is not limited to molecular beacon probe, strand displacement probes, hybridization probe or any probe that can be used in enzyme assisted target recycling.

5 In an embodiment, the oligonucleotide probe is molecular beacon probe comprising single strand nucleotide in a step-loop hairpin structure having fluorophore and quencher at each end of the strand. The fluorophore employed in the oligonucleotide probe have sufficient spectral overlap to ensure efficient quenching.

10 The fluorophore is selected from a group comprising FAM, HEX, TET, Cy3, Texas Red, ROX, TMR, LC Red 640, LC red 750 and Cy5. The quencher is selected from a group comprising Dabcyl, BHQ1, BHQ2, BHQ3, Iowa Black RQ, Iowa Black FQ, QSY7 and QSY 21.

15 In an embodiment, the molecular beacon probe comprises a fluorophore at the 5' end and quencher is at an internal position, at the end of the stem of the molecular beacon with an overhang of about 5 nucleotides to 10 nucleotides, preferably about 7 nucleotides that provides an open 3' end for action of the enzyme (illustrated in figure 6).

20 In an embodiment, the molecular beacon is designed to target a specific region of about 22 bases in length of the amplicon, wherein the GC content of the beacon is about 40% to 60%, the melting temperature of stem and loop of the beacon is about 60°C to 75°C with loop melting temperature higher than stem to favour amplicon-probe hybridization. Further, the molecular beacon comprises nucleotide T in specific position of 15th base, nucleotide G not adjacent to fluorophore. The molecular beacon employed in the method does have self-complementarity
25 or low risk of secondary structure.

In an embodiment, the oligo nucleotide probe is set forth as SEQ ID No. 15 (pf probe), SEQ ID No. 16 (CTXM probe), SEQ ID No. 17 (16s probe), SEQ ID No. 18 (16s probe_1), SEQ ID No. 19 (16s probe_2), SEQ ID No. 20 (16s probe_3) and SEQ ID No. 21 (16s probe_4).

30 In an embodiment, the GC content of SEQ ID No. 17 (16s probe), SEQ ID No. 18 (16s probe_1), SEQ ID No. 19 (16s probe_2), SEQ ID No. 20 (16s probe_3) and SEQ ID No. 21 (16s probe_4), is about 51.72%, about 44.83%, about 58.62%, about 48.28% and about 44.83%, respectively.

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In an embodiment, the melting temperature of SEQ ID No. 17 (16s probe), SEQ ID No. 18 (16s probe_1), SEQ ID No. 19 (16s probe_2), SEQ ID No. 20 (16s probe_3) and SEQ ID No. 21 (16s probe_4), is about 71.9°C about 68.9°C, about 74.9°C, about 69.9°C and about 68.9°C, respectively.

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In an embodiment, the stem melting temperature of SEQ ID No. 17 (16s probe), SEQ ID No. 18 (16s probe_1), SEQ ID No. 19 (16s probe_2), SEQ ID No. 20 (16s probe_3) and SEQ ID No. 21 (16s probe_4), is about 20°C about 18°C, about 22°C, about 20°C and about 18°C, respectively.

10

In an embodiment, the stem melting temperature of SEQ ID No. 17 (16s probe), SEQ ID No. 18 (16s probe_1), SEQ ID No. 19 (16s probe_2), SEQ ID No. 20 (16s probe_3) and SEQ ID No. 21 (16s probe_4), is about 28°C about 24°C, about 26°C, about 26°C and about 24°C, respectively.

15

In an embodiment, hybridizing the amplicon and the probe, followed by cleaving the probe by the enzyme is carried out in buffer and reagent, wherein the buffer is selected from a group comprising magnesium chloride, magnesium sulphate, magnesium acetate, potassium chloride, potassium sulphate, potassium acetate, sodium chloride, sodium sulphate, sodium acetate, Tris HCL and DTT; and the reagent is selected from a group comprising BSA and PEG.

20

In another embodiment, the hybridizing the amplicon and the probe, followed by cleaving the probe by the enzyme is carried out in buffer, wherein the buffer is selected from a group comprising magnesium chloride, magnesium sulphate, magnesium acetate, potassium chloride, potassium sulphate, potassium acetate, sodium chloride, sodium sulphate, sodium acetate, Tris HCL and DTT.

25

In an embodiment, the hybridizing the amplicon to the probe is carried out at a temperature of about 25°C to 40°C for a period of about 5 minutes to 10minutes. Further, the hybridized amplicon and the probe is contacted with the enzyme and cleaving of the probe by the enzyme is carried out at a temperature of about 25°C to 40°C, for a period of about 35 minutes to 90 minutes.

30

In an embodiment, in the method of the present disclosure amplification of the nucleic acid to obtain the amplicon is primary amplification. And, amplification of the signal by cleaving with the enzyme is secondary amplification.

35

The disclosure further relates to microplate having wells comprising primers, enzymes, reagents, probes and nucleoside triphosphate.

5 In an embodiment, the microplate conducts the method of the present disclosure, wherein the microplate is capable of performing primary amplification and secondary amplification for detection of nucleic acid in the sample, wherein the primary amplification includes but is not limited to polymerase chain reaction, ligase chain reaction, loop-mediated isothermal amplification, nucleic acid sequence based amplification, simple amplification based assay, helicase dependent amplification and rolling circle amplification. Preferably, the primary
10 amplification is loop-mediated isothermal amplification. The secondary amplification includes but is not limited to enzyme assisted target recycling.

In an embodiment, the microplate of the present disclosure has 6, 12, 24, 48, 96, 384, 1536, 3456 or 9600 wells, wherein the wells in the microplate are well differentiated to accommodate
15 the contents of the primary amplification and secondary amplification to perform the method of the present disclosure to detect nucleic acid in the sample.

In an embodiment, in the microplate of the present disclosure, the wells dedicated for primary amplification comprises primers, enzymes, buffers and nucleoside triphosphates. The primers
20 are dried with or without stabilizing agents such as trehalose or lyophilized. The enzymes are also dried in the presence of stabilizing agent such as trehalose and/or glycerol or alternatively the enzymes are crystallized as a micro crystal and maintained as dry powder. The nucleoside triphosphates are also dried along with enzymes in the presence of stabilizing agent such as trehalose and glycerol. The reagents are also dried in the presence of stabilizing agent such as
25 trehalose.

In an embodiment, each well in the microplate dedicated for primary amplification comprises primes, enzymes, reagents and nucleoside triphosphates, wherein the primers, enzymes, reagents and nucleoside triphosphate are dried in the presence of stabilizing agent such as trehalose or without any stabilizing agents. Alternatively, the enzymes are crystallized as a
30 microcrystal and added as dry powder.

In another embodiment, in the microplate of the present disclosure, the wells dedicated for secondary amplification comprises probes, enzymes and reagents. The probes are dried in the presence of stabilizing agent such as trehalose or lyophilized or without any stabilizing agent.
35 The enzymes are dried in the presence of stabilizing agent such as trehalose or alternatively the

enzymes are crystallized as a micro crystal and maintained as dry powder. The reagents are also dried in the presence of stabilizing agent such as trehalose and glycerol.

In another embodiment, each well in the microplate is dedicated for secondary amplification comprises probes, enzymes and reagents, wherein the probes, enzymes and reagent are dried in the presence of stabilizing agent such as trehalose or without any stabilizing agent. Alternatively, the enzymes are crystallized as micro crystal and added as powder.

In an embodiment, the microplate serves as storage of primers, probes, enzymes, buffers and nucleoside triphosphates. In order to activate the microplate to conduct the method of the present disclosure, predetermined amount of water including but not limited to nuclease free water and double distilled water or buffer or buffer and water is added to the wells and the contents are mixed. The mixed contents are transferred to a tube/vial for the primary amplification, followed by secondary amplification.

In another embodiment, the microplate is directly used for conducting the primary amplification and secondary amplification as described in the methods of the present disclosure, wherein predetermined amount of water including but not limited nuclease free water and double distilled water or buffer or buffer and water is added to the wells and the contents of the well are mixed. To the mixed contents the DNA sample is added and the microplate is placed in an appropriate device capable of supporting nucleic acid amplification, preferably in an isothermal condition. Once, the nucleic acid amplification (primary amplification) is completed, part or all the contents from the well dedicated to primary amplification is transferred manually by technique including but not limited to pipetting, to the wells dedicated to secondary amplification. Predetermined amount of water including but not limited to nuclease free water and double distilled water or buffer and water including but not limited to nuclease free water and double distilled water is added to the wells, followed by mixing to initiate secondary amplification.

In another embodiment, there is no manual transfer of contents from the wells dedicated to primary amplification to the wells dedicated to secondary amplification. Instead, there is automated transfer of the contents from the wells dedicated to primary amplification to the wells dedicated to secondary amplification, at a predetermined time point.

The flow of contents from the wells dedicated to primary amplification to the wells dedicated to secondary amplification is actuated by a pump, or by gravity or by pressure in a valve-less flow system.

- 5 In an embodiment, the present disclosure further relates to a kit comprising microplate having 6, 12, 24, 48, 96, 384, 536, 3456 or 9600 wells dedicated for primary amplification and secondary amplification, optionally along with buffer or water including but not limited to nuclease free water and double distilled water, or both.
- 10 In another embodiment, the microplate in the kit having wells comprises primers, probes, enzymes, reagents and nucleoside triphosphate, capable of conducting primary amplification and secondary amplification, respectively as described in the method of the present disclosure to detect nucleic acid in a sample.
- 15 Additional embodiments and features of the present disclosure will be apparent to one of ordinary skill in art based upon the description provided. The embodiments provide various features and advantageous details thereof in the description. Descriptions of well-known/conventional methods and techniques are omitted to not unnecessarily obscure the embodiments. The examples provided herein are intended merely to facilitate an understanding
- 20 of ways in which the embodiments provided may be practiced and to further enable those of skill in the art to practice the embodiments provided. Accordingly, the following examples should not be construed as limiting the scope of the embodiments.

EXAMPLES

- 25 **EXAMPLE 1: Detection of 16s rRNA gene from bacterial DNA using the methods of the present disclosure.**

EATR probes (SEQ ID Nos. 17-21) are designed for a conserved region of 16s rRNA of *Escherichia coli* (E. coli). LAMP primes (SEQ ID Nos. 5, 6, 11, 12, 13 and 14) are designed

30 to flank the EATR probes.

Using these primers, a primary amplification (LAMP) is carried out on a mixture of DNA sample from a uropathogenic strain of E. coli and normal (uninfected) human DNA as mock sample representing DNA extracted from urine. Therefore, the amount of human DNA used is

35 about 2500 copies/ μ l, representing the amount of DNA present in average normal urine sample.

In the sample containing human DNA, varying amounts of E. coli DNA is spiked (about 10 copies/ μ l, about 100copies/ μ l and about 1000copies/ μ l) representing low to high levels of infection. Figure 2a illustrates the output from the primary amplification using negative control (no DNA-NTC), about 10 copies/ μ l and about 100 copies/ μ l.

5

About 5 μ l of reaction output from the above described primary amplification (LAMP) is used in secondary amplification (EATR). During EATR, about 10nm to 100nm probe, about 4 units to 16 units of exonuclease III enzyme and buffer containing Tris-HCL, Bis-Propane, MgCl₂, DTT is added, thereafter water is added to make up volume to 100ul. Figure 2b illustrates the fold change overtime in the secondary amplification with EATR.

10

Inference: Primary amplification for about 1 hour shows specific amplification in the samples containing DNA. In the negative controls, either no amplification or non-specific amplification is seen. However, during secondary amplification, only the specific amplicons from the primary amplification are amplified and this leads to no non-specific amplification. Moreover, in about 20 minutes of secondary amplification, peak fold change is achieved, thereby effectively detecting the DNA in the sample.

15

EXAMPLE 2: Illustrating the synergistic effect of combination of Primary amplification with Secondary amplification.

20

The mock urine sample is created as described in Example 1.

Non-specific amplification (primary amplification) is induced in 4 of the 8 negative controls by increasing the concentration of Mg in the buffer mixture, which is known to cause non-specific amplification. Non-specific amplification, which can occur at random is also observed in 1 of the 4 negative controls in which the Mg is at optimal concentration.

25

The output (amplicons) of the primary amplification is subjected to secondary amplification, all 8 NTCs showed only basal signals with negative controls. Clearly demonstrating the ability of the secondary amplification in combination with primary amplification to prevent non-specific amplification from being detected, thereby reducing the risk of having false positives.

30

EXAMPLE 3: Detection of antimicrobial resistance genes by the method of the present disclosure.

EATR probe (SEQ ID No. 16) is designed for a conserved region of CTX-M-15 gene, which is responsible for resistance of beta lactam antibiotics such as cephalosporins. CTX-M-15 has

been implicated in antibiotic resistance of *E. coli* and many other organisms. LAMP primers (SEQ ID Nos. 3, 4, 9 and 10) are designed to flank the EATR probe target region.

Using the LAMP primers, a primary amplification (LAMP) is carried out on DNA extracted from a uropathogenic strain of *E. coli* confirmed through PCR and sequencing to carry the CTX-M-15 gene. The DNA from the *E. coli* strain and normal (uninfected) human DNA are mixed together to serve as mock samples representing DNA extracted from urine. Therefore, the amount of human DNA used is about 2500 copies/ μ l, representing the amount of DNA present in average normal urine samples. In the sample containing human DNA, varying amounts of *E. coli* DNA is spiked (about 10 copies/ μ l and about 1000 copies/ μ l) representing low to high levels of infection.

Figure 4 illustrates the result of the experiment, wherein the combination of primary amplification (LAMP) and secondary amplification (EATR) is clearly able to detect CTX-M-15 in a sample having as low as about 10 copies/ μ l of DNA. The detected 10 copies/ μ l would mean that the detected DNA is much lower than the copies of plasmid DNA carrying the CTX-M-15 gene, inferring that the method of the present disclosure is effectively sensitive and accurate.

EXAMPLE 4: Detection of *Plasmodium falciparum* in crude blood lysate by the method of the present disclosure.

EATR probe: SEQ ID No. 15 and LAMP primers: SEQ ID No. 1, 2, 7 and 8, were designed and employed in this experiment.

About 180 μ l of whole blood and 180 μ l of extraction buffer are mixed together by vortexing for about 10 seconds and boiled at a temperature of about 95°C for about 5 minutes. Samples are centrifuged at room temperature for about 3 minutes at about 10,000g rpm and clear supernatant of about 200 μ l is taken out. To the supernatant various quantities of *P. falciparum* DNA (about 5 copies/ μ l, 50 copies/ μ l and 100 copies/ μ l) is added, respectively. The sample with *P. falciparum* DNA is subject to primary amplification and secondary amplification. Negative control (NTC), positive control and a comparison with pure DNA mix of about 5 copies/ μ l of *P. falciparum* and human DNA are included in the method.

Figure 5 illustrates that the method of the present disclosure is able to detect *P.falciparum* in crude lysate having as low as 5 copies/ μ l *P.falciparum* DNA.

EXAMPLE 5: Comparison of method of the present disclosure and polymerase chain reaction for detecting nucleic acid.

DNA was extracted using Qiagen miniAMP DNA kit and stored in Eppendorf tubes at room temperature. A quantity of 2 to 5 μ l of DNA was used for PCR and similar quantity was used in the dual amplification of LAMP-EATR. PCR was carried out with the outer primers (Pf-F3 and Pf-B3) of LAMP and the limit of detection was 100 copies/ μ l. In the dual amplification, 10 copies/ μ l were detected, illustrated in figure 7. This clearly demonstrated the value of the dual amplification over PCR. Dual amplification with LAMP-EATR prevents the nonspecific amplification.

Further to validate the efficacy of the method of the present disclosure, the DNA extracted from about 10 malarial samples was subjected to the combination of the primary amplification and secondary amplification described in the present disclosure, illustrated in figure 8

EXAMPLE 6: Illustration of pre-loading components for secondary Amplification.

Pre-loading of probes, reagents and enzymes for secondary amplification is done by drying under vacuum conditions in the presence of stabilizers such as trehalose. Trehalose is known to impart stability to DNA probes such as Taqman probes and enzymes.

In order to detect 16s rRNA, the EATR molecular beacon probe is dried under vacuum in the presence of varying amount of trehalose. With about 0.67mM trehalose, the probe is stable for about 25 days at 27°C. However, with accelerated testing the probe is stable for about 6 days at 47°C. Table 1 illustrates the effect of varied concentration of trehalose on stability of the probes.

At the same time, the background fluorescence from the probe dried without trehalose showed significant rise in signal while probe dried with trehalose showed no change in signal

Results so far: EATR MB

		Day 0	Day 6	Day 12
Fold Increase	In-solution MB	23.26	22.06	26.68
	0.67M	21.89257	22.45803	22.33462
	0.33M	23.64949	21.03305	18.21608
	0.17M	22.32074	21.63724	20.43838
	0.08M	22.33	21.52863	19.33722
	0.04M	21.93	22.62863	18.41542
	0.0M	22.62	9.355166	4.529686

Background buffer + Probe	Week 0	Week 1	Week 2
0.0M MB	18.455	31.057	51.425
0.67M MB	18.6075	18.87	18.8815

For optimum stabilization of MB, Trehalose conc of 0.67M or higher is required

Table 1: Effect of varied concentration of Trehalose on stability of probe.

EXAMPLE 7: Preparation of the kit

- 5 Microplate was prepared in a polymer material (comprising of one of acrylic or polypropylene). A block of the material measuring in thickness (2mm or 5mm or 10mm) and a width of 1cm. Wells were drilled and CNC machined for a smooth finish or laser cut. Well dimensions were determined to hold about 10µl to 25µl LAMP contents such as enzyme, buffer, primers and reagents and about 100µl to 200µl EATR contents such as oligonucleotide probe, enzyme, buffer. In each row, well for LAMP and EATR were made to ensure ease of transfer.

Master mix for LAMP was prepared as a solution or in dehydrated or lyophilized format and added to the LAMP well. EATR master mix was prepared as a solution or in dehydrated or lyophilized format and added to the EATR well

15 Microplate was then covered with a detachable cover plated and stored in a sealed container with silica gel at room temperature or at 4°C, along with buffer or water or both.

WE CLAIM:

1. A method for detecting nucleic acid, said method comprises steps of-
contacting sample comprising the nucleic acid with the nucleic acid amplification
reaction mixture, followed by amplifying the nucleic acid to obtain amplicon;
hybridizing the amplicon and oligonucleotide probe; and
contacting the hybridized amplicon and the probe with enzyme and cleaving the
probe by the enzyme to release the amplicon, wherein the cleaved probe produces
signal, thereby detecting the nucleic acid.
2. The method of detecting the nucleic acid as claimed in claim 1, wherein the method
further comprises hybridizing the released amplicon with another oligonucleotide
probe, followed by cleaving the probe by the enzyme to release the amplicon, wherein
the cleaved probe produces signal.
3. The method of detecting the nucleic acid as claimed in claim 1, wherein the nucleic
acid amplification reaction mixture comprises-
 - a. Primer selected from a group comprising outer primer, inner primer, middle primer
and loop primer, or any combinations thereof;
 - b. Enzyme selected from a group comprising Taq polymerase and Bst polymerase;
 - c. Buffer selected from a group comprising magnesium chloride, magnesium sulphate,
magnesium acetate, potassium chloride, potassium sulphate, potassium acetate,
sodium chloride, sodium sulphate, sodium acetate, Tris HCL and DTT; and
 - d. Reagent selected from a group comprising poly ethylene and dNTPs.
4. The method of detecting the nucleic acid as claimed in claim 3, wherein the melting
temperature of the outer primer, middle primer and loop primer is about 55°C to 61°C,
and the melting temperature of the inner primer is about 62°C to 66°C.
5. The method of detecting the nucleic acid as claimed in claim 1, wherein the
amplification of the nucleic acid is carried for a period of about 30 minutes to 90
minutes at temperature ranging from about 60°C to 70°C, and for a period of about 5
minutes to 10 minutes at temperature ranging from about 25°C to 40°C.

6. The method of detecting the nucleic acid as claimed in claim 1, wherein the amplicon is about 300bp to 400bp.
7. The method of detecting the nucleic acid as claimed in claim 1 or 2, wherein the oligo
5 nucleotide probe is selected from a group comprising molecular beacon probe, strand displacement probe and hybridization probe; wherein the oligonucleotide probe comprises fluorophore selected from a group comprising FAM, HEX, TET, Cy3, Texas Red, ROX, TMR, LC Red 640, LC red 750 and Cy5 and quencher selected from a group comprising Dabcyl, BHQ1, BHQ2, BHQ3, Iowa Black RQ, Iowa Black FQ, QSY7 and
10 QSY 21; and the oligonucleotide probe comprises GC content of about 40% to 60% and the melting temperature of the oligonucleotide probe is about 60°C to 75°C.
8. The method of detecting the nucleic acid as claimed in claim 1 or 2, wherein the
15 hybridizing the amplicon to the probe is carried out at a temperature of about 25°C to 40°C for a period of about 5 minutes to 10 minutes and the cleaving of the probe by the enzyme is carried out at a temperature of about 25°C to 40°C, for a period of about 35 minutes to 90 minutes.
9. A kit comprising microplate and buffer or water or combination thereof, wherein the
20 microplate comprises primers, oligonucleotide probes, enzyme and reagent.
10. The kit as claimed in claim 9, wherein the microplate has dedicated wells for carrying out primary amplification and secondary amplification, respectively.
- 25 11. The kit as claimed in claim 9, wherein the primer is selected from a group comprising outer primer, inner primer, middle primer and loop primer, or any combinations thereof; enzyme is selected from a group comprising Bst polymerase-Bst 2.0, Bst polymerase-Bst 3.0, Bst large fragment polymerase, ,GspSSD LF DNA polymerase (from OptiGene) and OmniAmp (from Lucigen),; buffer is selected from a group comprising
30 magnesium chloride, magnesium sulphate, magnesium acetate, potassium chloride, potassium sulphate, potassium acetate, sodium chloride, sodium sulphate, sodium acetate, Tris HCL and DTT; reagent is selected from a group comprising poly ethylene and dNTPs; and oligonucleotide probe is selected from a group comprising molecular beacon probe, strand displacement probe, hybridization probe, wherein the
35 oligonucleotide probe comprises fluorophore selected from a group comprising FAM,

HEX, TET, Cy3, Texas Red, ROX, TMR, LC Red 640, LC red 750 and Cy5; and quencher selected from a group comprising Dabcyl, BHQ1, BHQ2, BHQ3, Iowa Black RQ, Iowa Black FQ, QSY7 and QSY 21.

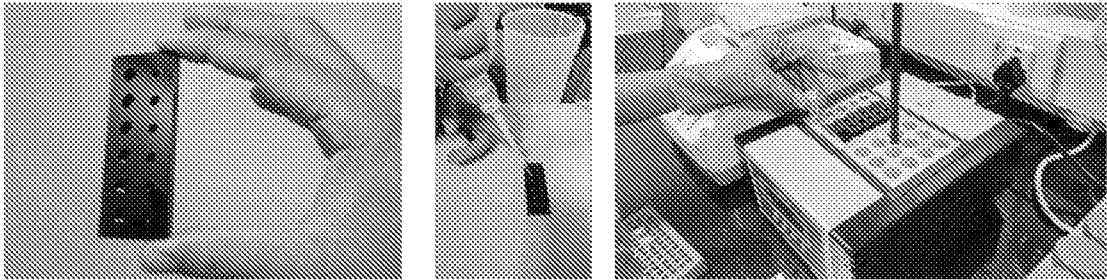


FIGURE 1

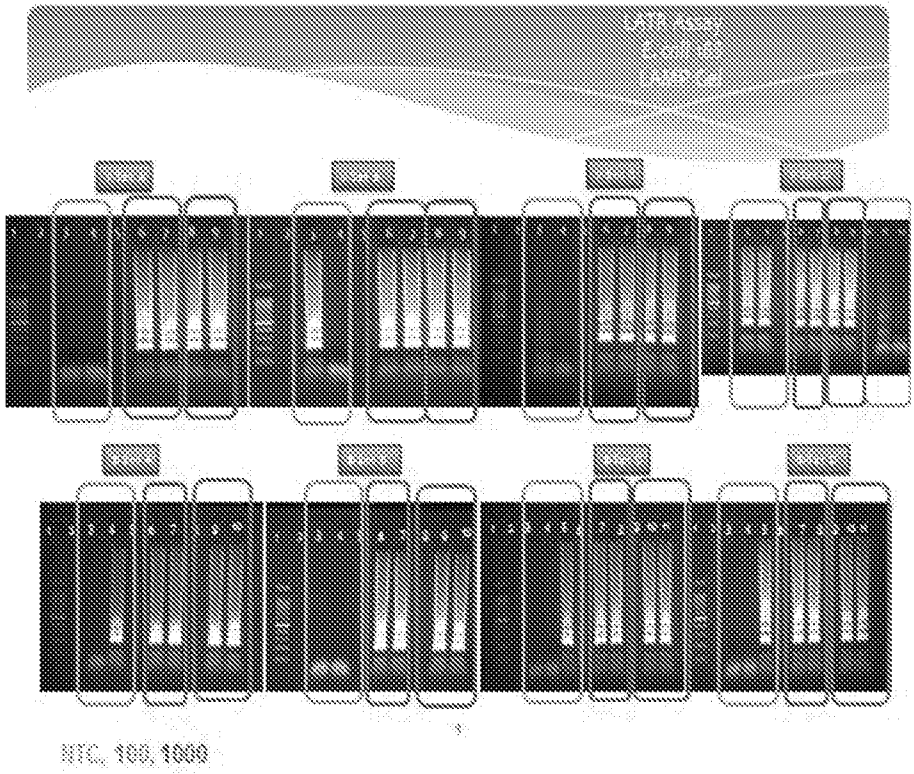


FIGURE 2a

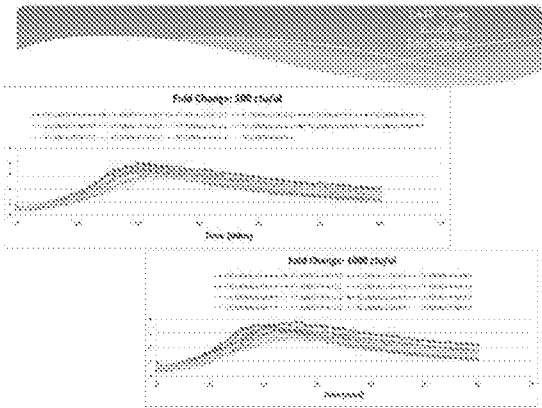


FIGURE 2b

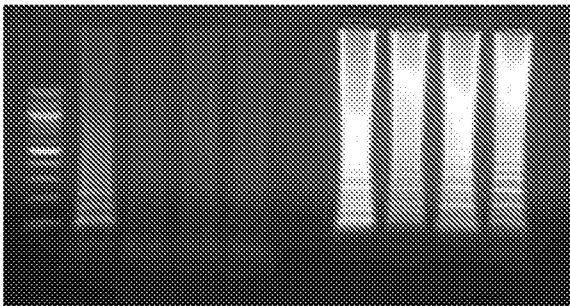


FIGURE 3a

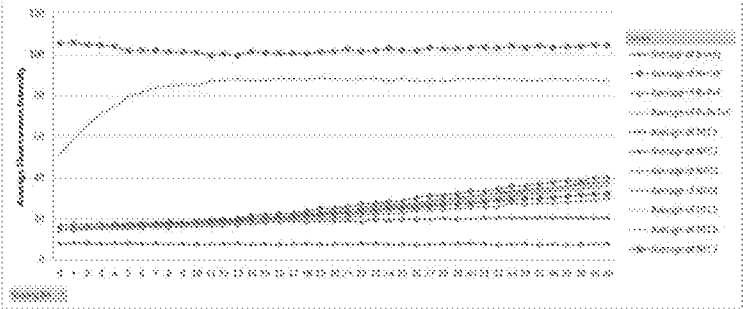


FIGURE 3b

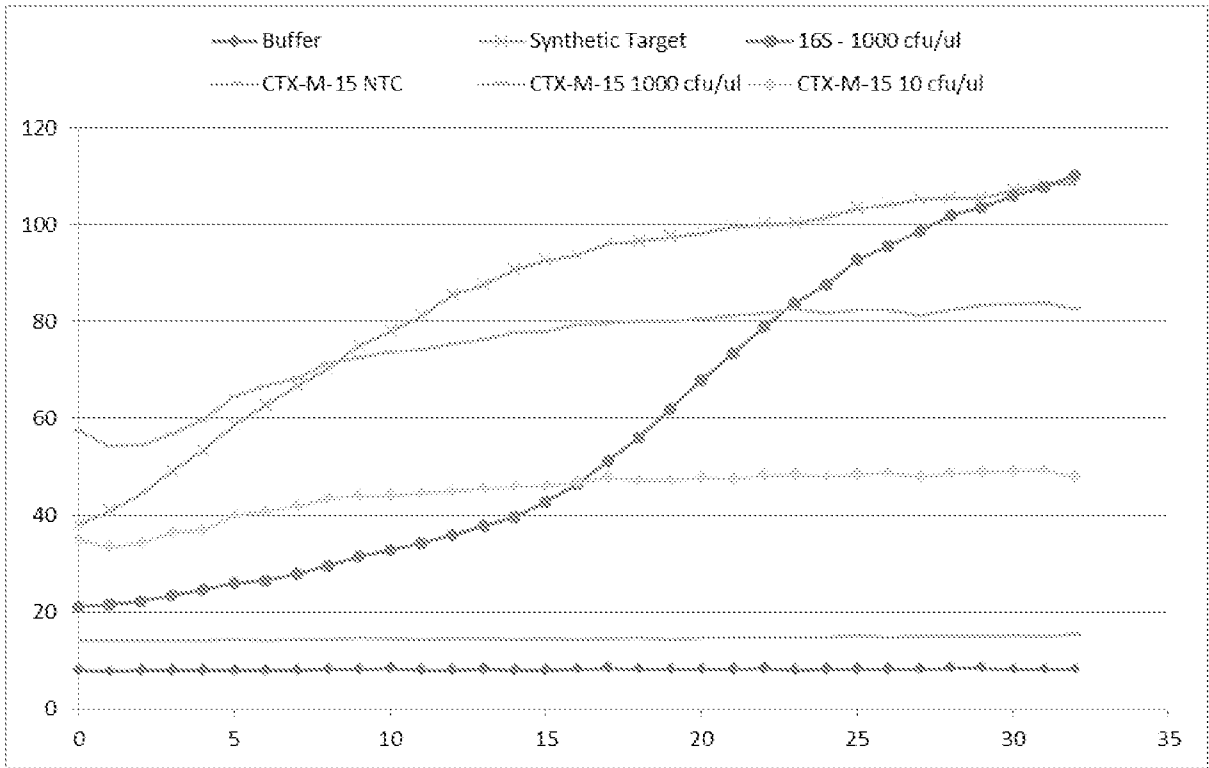


FIGURE 4

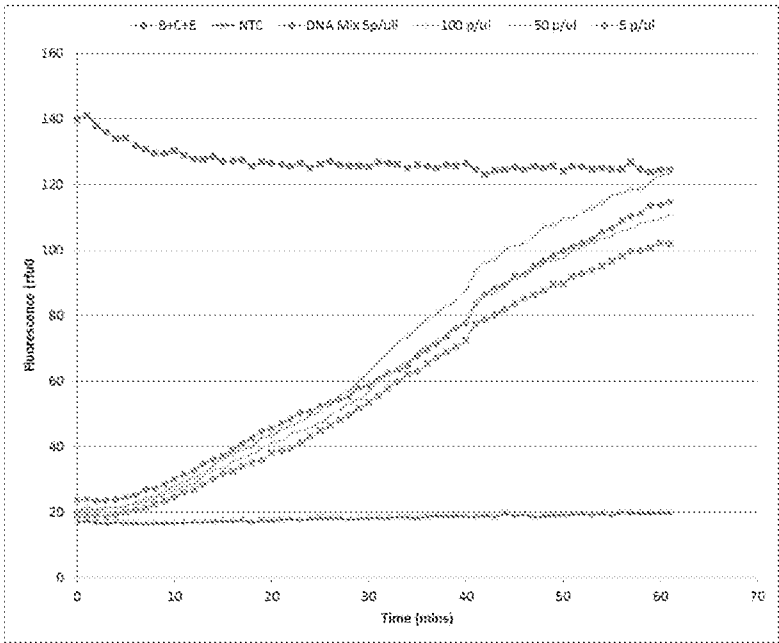


FIGURE 5

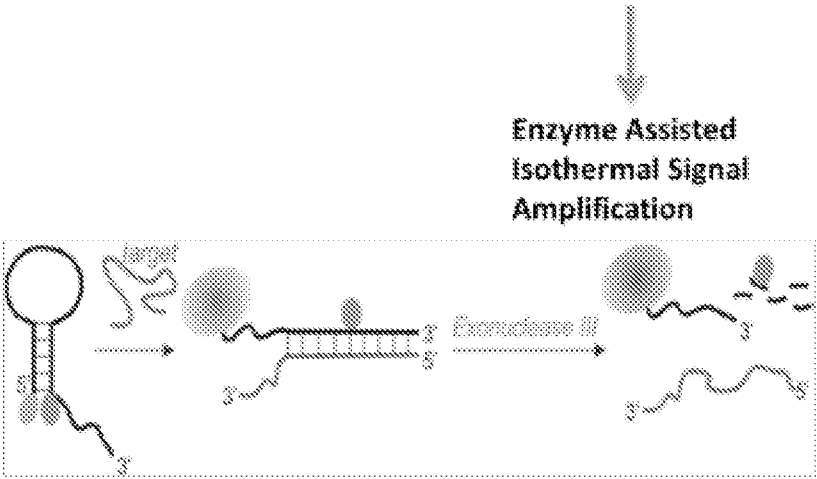
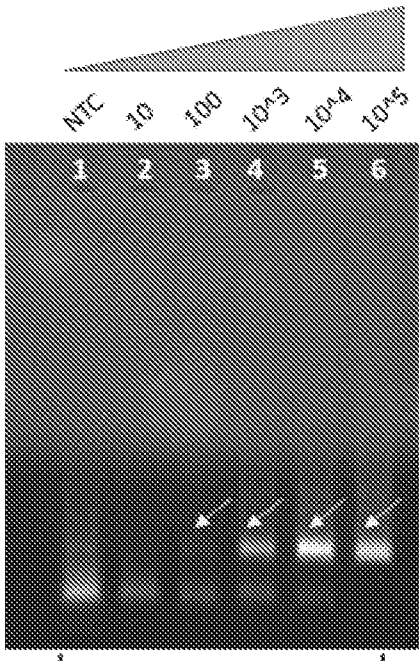
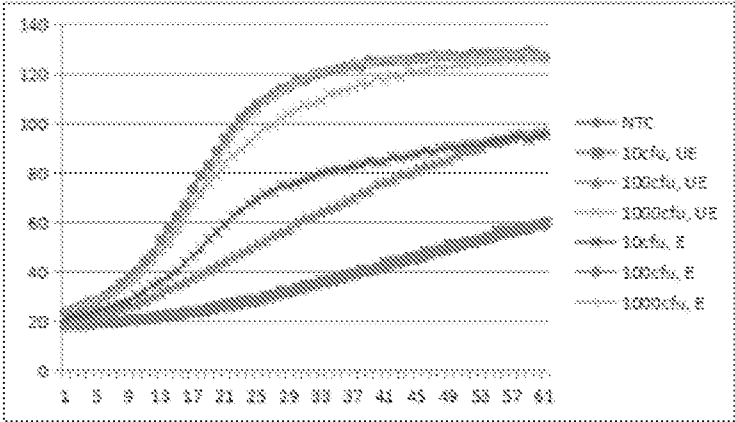


FIGURE 6

PCR with FIP/BIP



a



b

FIGURE 7

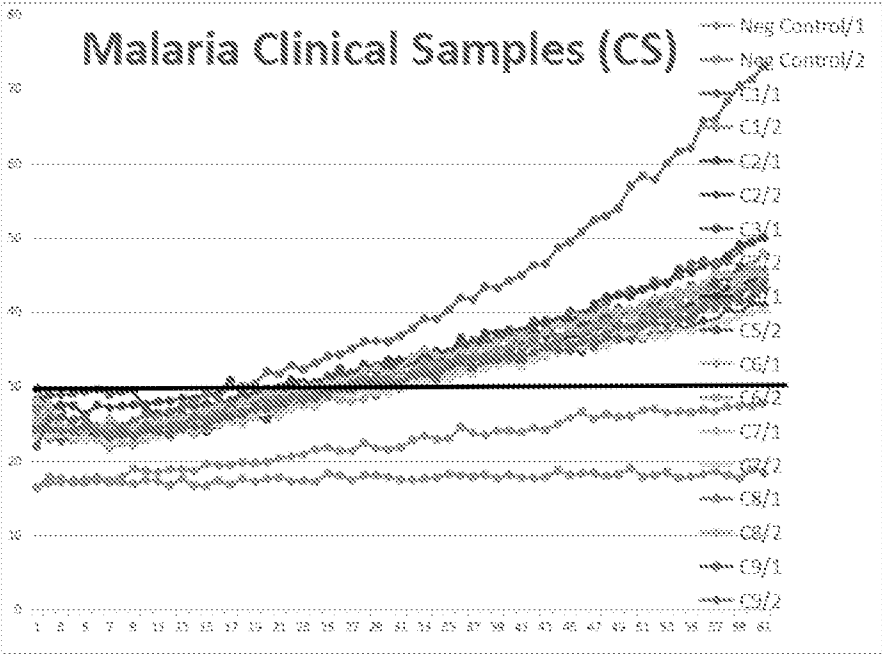


FIGURE 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2017/057387

A. CLASSIFICATION OF SUBJECT MATTER
C12Q1/68, C07H21/00 Version=2018.01

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, C07H

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)

Patseer, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Gerasimova YV, Kolpashchikov DM. Enzyme-assisted target recycling (EATR) for nucleic acid detection. Chemical Society Reviews. 2014;43(17):6405-38. See: Abstract, Fig:1,2,9-11, material and methods -----	1-11
Y	Nagdev KJ, Kashyap RS, Parida MM, Kapgate RC, Purohit HJ, Taori GM, Dagainawala HF. Loop-mediated isothermal amplification for rapid and reliable diagnosis of tuberculous meningitis. Journal of clinical microbiology. 2011 May 1;49(5):1861-5. See: Abstract, methods, Table 1-2, Fig:1, Results	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"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
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"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	

Date of the actual completion of the international search

23-02-2018

Date of mailing of the international search report

23-02-2018

Name and mailing address of the ISA/

Indian Patent Office
Plot No.32, Sector 14, Dwarka, New Delhi-110075
Facsimile No.

Authorized officer

Akash Kumar

Telephone No. +91-1125300200