

NUCLEIC ACID DETECTION METHOD USING LAMP AND PROBE DETECTION

Field of the Invention

The invention lies in the field of nucleic acid amplification and detection, in particular isothermal nucleic acid amplification and the detection of the amplicons.

Background

Loop-mediated isothermal amplification (LAMP) is a unique isothermal amplification method having a number of advantages (See, e.g., US Patent No. 6,410,278 B1 and US Patent No. 7,374,913 B2).

Firstly, it is an isothermal nucleic acid amplification method, which eliminates the need for a specific device to control the temperature cycling, such as those needed for PCR, and thus it is ideally suited for point-of-care testing. Secondly, LAMP can generate a large amount of amplicon within a short period of time with high specificity. These two advantages enable relatively easy detection and a potential wide application in point-of-care testing. Another advantage is that, compared with other isothermal amplification methods, LAMP only requires one enzyme, making the system easier to handle.

However, despite these advantages, there are two major hurdles when LAMP is employed for various applications. Firstly, due to the high number of primers (4 to 6 primers vs. 2 primers for regular PCR) and fast amplification nature, non-specific amplification mainly arising from primer dimers is often observed. This is a severe obstacle when it comes to new target development, as each new target requires tedious optimization of reaction conditions, often requiring sacrificing assay sensitivity or/and speed. Secondly, multiplexing poses a significant challenge as multiple primers are needed for each target of interest and target specific detection methods are required.

Several probe-mediated detection methods have been developed to solve these problems. However, to date all existing methods have certain limitations.

For example, it is known to achieve probe detection through labelling of primers either directly (CN 105950778 A and Higgins et al 2018, Int. J. Mol. Sci 19 (2), 524) or through binding to another complementary probe (US Patent Application No. 2013/0171643 A1 and US Patent No. 9,074,249 B2). These methods have the drawback that they often lead to false positive results due to primer self-extension in the absence of the target of interest.

In other approaches, loop primers or loop regions are used for probe design (See, e.g., EP 1795612 A1, US Patent No. 10,072,309 B1, JP 4516032 B2, and Yaren et al. (2016), J Virol Methods 237:64-71). While these methods greatly eliminate the issue of false positives, they suffer from other drawbacks. In the method described in European patent application 1795612 A1 fluorescent labelled probe in combination with PEI are utilized to detect amplification through the PCR product precipitation. However, this method is very difficult to conduct multiplexing reactions. In addition, a lot

of effort is needed for molecular beacon design to obtain optimal signal/noise ratio (US Patent No. 10,072,309 B1 and Yaren et al. (2016), J Virol Methods 237:64-71). Moreover, in all of the above mentioned methods, each unit of amplicon can only generate one unit of signal, thus limiting detection sensitivity and speed.

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To overcome these problems, the inventors of the present invention have developed a novel method that combines LAMP with cycling probe detection. This new method achieves an easy assay design process and higher specificity, signal and speed. The new method also provides for an easier multiplexing solution using the LAMP method.

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Summary of the Invention

In a first aspect, the present invention relates to a method for determining the presence or amount of a target nucleic acid molecule in a sample using loop mediated isothermal amplification (LAMP), the method comprising:

- 15 (a) combining a LAMP reaction mixture, a DNA polymerase with strand displacement properties, a sample containing the target nucleic acid molecule, at least one detection probe, and at least one cleaving agent;
wherein the LAMP reaction mixture comprises a LAMP primer set of 4 to 6 primers, comprising two inner primers (FIP and BIP), two outer primers (F3 and B3), and optionally one or two loop primers (LF
20 and/or LB); and
wherein the at least one detection probe is a single stranded probe comprising a DNA sequence complementary to a sequence in a loop region of the amplicons formed by the LAMP reaction that can hybridize to said amplicons under LAMP assay conditions and form a double-stranded probe:target complex, the at least one detection probe comprising a scissile linkage in the DNA sequence
25 complementary to a sequence in a loop region of the amplicons formed by the LAMP reaction, wherein said scissile linkage in the probe:target complex but not the non-hybridized probe is cleavable under LAMP assay conditions;
wherein under LAMP assay conditions the at least one cleaving agent is capable of cleaving the at least one detection probe at said scissile linkage when said probe is hybridized to its target, wherein
30 upon cleavage of said at least one detection probe at the scissile linkage the fragments of said at least one detection probe are released from the probe:target complex so that the cycle of detection probe binding, cleavage of the probe and release of the probe fragments can be repeated;
- (b) amplifying the target nucleic acid molecule by LAMP under suitable assay conditions that allow generation of the target amplicons by the LAMP reaction and multiple cycles of: (i) hybridization of the
35 at least one detection probe to the amplicons formed by the LAMP reaction, (ii) cleavage of the detection probe in the formed probe:target complex, and (iii) release of the cleaved probe fragments from the target; and
- (c) detecting and optionally quantifying the released fragments of said at least one detection probe, and therefrom determining the presence or amount of the target nucleic acid molecule in the sample.

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In various embodiments of these methods, the two inner primers (FIP and BIP) each comprise a target complementary region on their 3' end (F2 and B2) and a target identical region on their 5' end (F1c and B1c), wherein, in the target nucleic acid, the sequence recognized by the target complementary region of the inner primers (F2c and B2c) lies 3' to the sequence identical to the target identical sequence on the 5' end of the inner primers (F1c and B1c). In such embodiments, the two outer primers can each comprise a target complementary region (F3 and B3), wherein in the target nucleic acid the sequence targeted by the target complementary region of the outer primers (F3c and B3c) is located 3' relative to the sequence of the target nucleic acid bound by the target complementary region of the inner primers (F2c and B2c).

In various embodiments of the methods described herein, the Forward Inner Primer (FIP) consists of a F2 region at the 3' end and a F1c region at the 5' end, wherein the F2 region is complementary to a region (the F2c region) of the template sequence and the F1c region is identical to a region (the F1c region) of the template sequence. In addition, the Backward Inner Primer (BIP) consists of a B2 region at the 3' end and a B1c region at the 5' end, wherein the B2 region is complementary to a region (the B2c region) of the template sequence and the B1c region is identical to a region (the B1c region) of the template sequence. In such embodiments, the Outer Forward Primer (also called F3 Primer) consists of a F3 region which is complementary to a region (the F3c region) of the template sequence and the Outer Backward Primer (also called B3 Primer) consists of a B3 region which is complementary to a region (the B3c region) of the template sequence.

Furthermore, the one or two optional loop primers may each comprise a target complementary region that targets a sequence of the amplicons formed between the target complementary region on the 3' end of the inner primers and the sequence complementary to the target identical sequence on the 5' end of the inner primers. These target complementary sequences of the loop primers each target a sequence of the amplicons formed in the LAMP reaction between the F1 and F2 and/or B1 and B2 region.

In various embodiments, the at least one detection probe has the structure

DNA1-R-DNA2,

wherein DNA1 and DNA2 are DNA sequences that are complementary to adjacent regions in the target nucleic acid molecule and R is the scissile linkage.

In various embodiments, the scissile linkage in the detection probe is cleavable chemically or enzymatically under LAMP assay conditions, only if the probe is hybridized to the target amplicon. In various embodiments, enzymes are preferred as cleaving agents, with said enzymes specifically recognizing the probe:target double-stranded hybrid over the single-stranded unpaired probe and cleaving only the probe and not the target strand. Such cleaving agents may be or include an endonuclease, a ribonuclease (also known as RNase), a nicking enzyme, and mixtures thereof. For

example, the endonuclease may be an AP endonuclease, a T4 endonuclease V, a methylation dependent nicking enzyme and mixtures thereof. The RNase may be RNase HI, RNase HII, RNase HIII, related enzymes and mixtures thereof.

- 5 In various specific embodiments, the scissile linkage comprises at least one RNA nucleotide. In such embodiments, the cleaving agent preferably comprises RNase H. Alternatively, the scissile linkage may comprise at least one apurinic/apyrimidinic site (AP site). In such embodiments, the cleaving agent preferably comprises an AP endonuclease. In various embodiments, the scissile linkage may comprise at least one deoxyribonucleotide with its base methylated or substituted and the cleaving agent comprises a specific endonuclease that recognizes the base modification.

- 10 In various embodiments, the released fragments are labeled with a detectable marker and labeled fragments are detected. The at least one detection probe may, for example, comprise a fluorescence resonance energy transfer (FRET) pair consisting of two fluorophores, two chromophores or a fluorophore/chromophore quencher pair. In such embodiments, the first member of the pair may be located on the 5' end of the probe or in the sequence 5' to the scissile linkage and the second member of the pair may be located on the 3' end of the probe or in the sequence 3' to the scissile linkage, wherein the members of the pair are located, i.e. spaced apart, such that they can interact in the intact non-cleaved probe and selected such that the fluorescence signal changes upon cleavage of the probe, i.e. in case both members are not in close proximity to each other, for example if they are part of different fragments after cleavage.

- 15 In various embodiments, the sequence in the target nucleic acid molecule/amplicon recognized by the target complementary region of the at least one detection probe is located between the sequence complementary to the sequence targeted by the target complementary region on the 3' end of the inner primers (F2 and B2) and the sequence complementary to the sequence identical to the target identical sequence on the 5' end of the inner primers (F1 and B1), i.e. between F2 and F1 or between B2 and B1. The sequence recognized by the at least one detection probe lies preferably 3' to the sequence in the target nucleic acid molecule targeted by the target complementary region of the loop primers, with the latter also being located between F2 and F1 or B2 and B1.

It can be preferred, in various embodiments, that the sequences targeted by the target complementary regions of the LAMP primers and the at least one detection probe are non-overlapping.

- 20 The methods of the invention may include the use of more than one detection probe for a single target nucleic acid molecule. In such embodiments, there are included at least two or more detection probes that target the same target molecule, preferably, however, at different and optionally also non-overlapping sequence regions of the same target molecule.

The methods of the invention may comprise multiplexing methods and may be used for determining the presence or amount of two or more target nucleic acid molecules in a sample, wherein such methods use one common LAMP primer set or separate LAMP primer sets and/or one or more detection probes for each target nucleic acid molecule or one probe for related target nucleic acid molecules.

In various embodiments, the target nucleic acid molecule may be a nucleic acid of a virus or bacterium or any other pathogen, optionally pathogenic for humans, including a bacterial, fungal, parasite or viral nucleic acid molecule or a cDNA reverse transcript of a bacterial, fungal, parasite or viral RNA, for example a cDNA of a dengue virus RNA, Zika virus, Chikungunya virus RNA, Yellow fever virus RNA, Japanese encephalitis virus RNA, influenza virus RNA, HIV RNA, or hepatitis C virus RNA.

In such methods, where the target nucleic acid is cDNA of dengue virus RNA (serotype 1 or serotype 3),

- (1) the LAMP inner forward primer may comprise the nucleic acid sequence set forth in SEQ ID NO:1, 2 or 3 (GB_T1_3UTR_FIP and GB_T1T3_3UTR_FIP and GB_T3_3UTR_FIP) or a variant thereof having at least 90 % sequence identity over the entire length;
- (2) the LAMP inner backward primer may comprise the nucleic acid sequences set forth in SEQ ID NO: 4 (GB_T1_3UTR_BIP) or a variant thereof having at least 90% sequence identity over the entire length;
- (3) the LAMP outer forward primer may comprise the nucleic acid sequence set forth in SEQ ID NO:5 (GB_T1_3UTR_F3) or a variant thereof having at least 90 % sequence identity over the entire length;
- (4) the LAMP outer backward primer may comprise the nucleic acid sequence set forth in SEQ ID NO:6 (GB_T1_3UTR_B3) or a variant thereof having at least 90 % sequence identity over the entire length;
- (5) the LAMP loop forward primer, if present, may comprise the nucleic acid sequence set forth in SEQ ID NO:7 or 8 (GB_T1_3UTR_LPF or GB_T1T3_3UTR_LPF) or a variant thereof having at least 90 % sequence identity over the entire length; and
- (6) the LAMP loop backward primer, if present, may comprise the nucleic acid sequence set forth in SEQ ID NO:9 (GB_T1_3UTR_LPB) or a variant thereof having at least 90 % sequence identity over the entire length.

In such methods, the at least one detection probe may comprise the nucleic acid sequence set forth in any one of SEQ ID Nos. 10-12 (T1 3UTR F Probe, T3 3UTR F Probe, T1_3UTR_B2LP_CP_rA) or a variant thereof having at least 90 % sequence identity over the entire length.

In another aspect, the invention is also directed to a kit for determining the presence or amount of a target nucleic acid molecule in a sample by loop mediated isothermal amplification (LAMP), the kit comprising:

- A. a LAMP reaction mixture;
- B. a DNA polymerase with strand displacement properties;
- C. at least one detection probe under conditions that allow the LAMP reaction to occur; and
- D. at least one cleaving agent for the at least one detection probe;

5 wherein the LAMP reaction mixture comprises a LAMP primer set of 4 to 6 primers, comprising two inner primers (FIP and BIP), two outer primers (F3 and B3), and optionally one or two loop primers (LF and/or LB), the primers being specific for the target nucleic acid molecule;

wherein the at least one detection probe is a single stranded probe comprising a DNA sequence complementary to a sequence in a loop region of the amplicons formed by the LAMP reaction that can
 10 hybridize to said amplicons under LAMP assay conditions and form a double-stranded probe:target complex, the at least one detection probe comprising a scissile linkage in the DNA sequence complementary to a sequence in a loop region of the amplicons formed by the LAMP reaction, wherein said scissile linkage in the probe:target complex but not the non-hybridized probe is cleavable under LAMP assay conditions and said cleavage leads to release of one or more fragments of said at least
 15 one detection probe adjacent to said scissile linkage from the probe:target complex; and
 wherein under LAMP assay conditions the at least one cleaving agent is capable of cleaving the at least one detection probe at said scissile linkage when said probe is hybridized to its target, wherein upon cleavage of said at least one detection probe at the scissile linkage the fragments of said at least one detection probe are released from the probe:target complex.

20 Such a kit may be used to carry out the methods described herein and may optionally also include user instructions, such as a manual, as well as various auxiliaries, including, for example, buffers and the like.

25 *Brief Description of the Drawings*

Figure 1 schematically shows two exemplary embodiments of a combination of the LAMP method with the cycling probe method. Upon generation of LAMP amplicons from the target strand in the said examples, the cycling probe binds to the back loop region, between B1 and B2 region, specifically to a region in the target that lies 3' relative to the back loop primer binding region. (The drawing only shows
 30 the binding of probe to the back-loop region; while the probe could similarly be designed to bind to the forward-loop region). In example 1, the cycling probe containing one or more ribonucleotides is cleaved by RNase H2 after binding to the LAMP amplicon. This relinquishes the quencher effect on fluorescence signal. Dissociation of the cut probe fragments from the LAMP amplicon allows the binding of another cycling probe. In example 2, the cycling probe is modified to include an abasic site
 35 (no nucleobase present) that, upon binding to the LAMP amplicon, will be recognized and cleaved by endonuclease IV, followed by release of the probe fragments and generation of fluorescence signal. Another cycling probe then binds the amplicon and the cleavage cycle is repeated.

40 Figure 2 (A): SYBR Green signal for LAMP coupled with cycling probe (Cy5 channel) reactions at 65 °C. The LAMP method was able to amplify 500 copies of plasmid DNA template consistently. Non-

specific amplification signals were detected after 12 mins. *Bst* 3.0 DNA Polymerase (NEB) and Isothermal Amplification Buffer II (IDT) were used in these reactions. (B) Cy5 signal (detection probe) for LAMP with cycling probe reactions as described herein. Cy5 fluorescence is released from probe cutting by RNase H2 when Cy5-labelled probe binds to LAMP amplicon. The time taken for Cy5 fluorescence to be detected was similar to that of SYBR Green. The non-specific amplification signals for the negative controls without target (NTC) were successfully suppressed.

Figure 3 (A) SYBR Green signal for isothermal amplification at 65 °C. The LAMP method was able to amplify 100 and 500 copies of plasmid DNA template consistently. Non-specific amplification signals were detected after 25 mins. (B) Isothermal amplification at 65 °C of plasmid DNA detected via Cy5 fluorescence released by cycling probe cutting. LAMP amplification coupled with fluorescent probe cutting performed similarly to SYBR Green intercalation. Additionally, non-specific amplification signals for the NTC were successfully suppressed.

Figure 4 (A) SYBR Green signal for RT-LAMP coupled with cycling probe at 65 °C. Both 7500 copies and 75000 copies of RNA templates were amplified consistently. Non-specific amplification signals were detected after 20 mins. (B) Cy5 signal for RT-LAMP with cycling probe reactions. Except for a slight delay, Cy5 signals were comparable to SYBR Green signals. The non-specific amplification signals for the NTC were suppressed.

Figure 5 (A) RT-LAMP amplification and serotype-specific cycling probe detection (T1 3'UTR F-FAM and T3 3'UTR F-Cy5) of 5000 copies of DENV1 (T1_+VE) or DENV3 (T3_+VE) RNA from FAM channel. Only signal from DENV1 RNA reaction was observed at around 15 mins of the amplification while the DENV3 RNA reaction did not show any significant fluorescence signal. There were no signals observed from NTC reactions. (B) RT-LAMP amplification and serotype-specific cycling probe detection (T1 3'UTR F-FAM and T3 3'UTR F-Cy5) of 5000 copies of DENV1 (T1_+VE) or DENV3 (T3_+VE) RNA from Cy5 channel. Only signal from DENV3 RNA reaction was observed at around 10 mins of the amplification while the DENV1 RNA reaction did not show any significant fluorescence signal. There were no signals observed from NTC reactions.

Figure 6 (A) Comparison of the Cy5 fluorescence signals generated by probe cutting versus probe binding to LAMP amplicons. In the presence of RNase H2, probe cutting resulted in a much higher fluorescence compared to probe binding alone (NEC; similar to the mechanism of molecular beacons or other binding only probes). (B) Comparison of the FAM fluorescence signals generated by probe cutting versus probe binding to LAMP amplicons. In the presence of RNase H2, probe cutting resulted in a much higher fluorescence compared to probe binding alone (NEC; similar to the mechanism of molecular beacons or other binding only probes). In this case (FAM fluorescence), probe binding alone did not result in fluorescence signals that can be readily differentiated from NTC background.

Figure 7 illustrates the LAMP general concept with 3 pairs of primers: a pair of inner primers (FIP and BIP), a pair of outer primers (F3 and B3) and a pair of loop primers (loop forward primer = LFP and loop backward primer = LBP). Each inner primer comprises a target complementary sequence (F2 in FIP and B1c in BIP) as well as a target identical sequence (F1c in FIP and B2 in BIP). During the LAMP process, the inner primer FIP hybridizes to F2c in the target sequence and performs complementary DNA strand synthesis. The outer primer F3, hybridizes to F3c in the target sequence and after initiating DNA synthesis, displaces the FIP-initiated complementary DNA strand. This latter DNA serves as a template for DNA synthesis by BIP and for strand displacement by B3. The resulting DNA is a dumb-bell shaped structure with two single-stranded regions (between F2c-F1c and B1-B2), wherein the single-stranded region between B1 and B2 can be bound by the (backward) loop primer (LB) to accelerate LAMP amplification. The forward loop primer may bind to the region between F1 and F2 on the complementary strand to the dumbbell structure being described.

Detailed Description

The present invention is based on the inventors' surprising finding that many of the existing drawbacks that prevent LAMP assays being widely used for target detection can be overcome by combination with a specific probe-based detection method. This provides for a simple, efficient and highly sensitive and specific method that allows point-of-care testing without expensive equipment.

The probe detection method used is also known as "cycling probe" technology. This technology is a probe signal amplification strategy, in which each probe will go through the following process: 1) binding to the target, 2) cleaving of the probe, and 3) dissociation of the two cleaved probe fragments from the template. Due to the dissociation of the probe fragment, this process enables recycling of the template and thus generates multiple signals for each single template, as the recycled template may be used for multiple cycles of probe binding, cleavage and dissociation. One known example of this method uses a chimeric DNA-RNA-DNA probe to detect complementary target DNA sequence (US Patent Application No. 5,011,769 A and International patent publication WO1995/014106 A2). Hybridization of chimeric probe to complementary target DNA forms a double-stranded hybrid that is recognized by the enzyme RNase H. RNase H cleaves the RNA portion of the duplex and the resultant cleaved probe fragments will dissociate from target DNA. Another intact probe will hybridize to the target DNA again and RNase H-mediated cleavage of probe will occur. Repeated cycles of probe hybridization to target DNA, RNase H cleavage and cleaved probe dissociation will result in probe/signal amplification. Multiplexing is also possible with the use of multiple differentially-labelled cycling probes targeting different sequences. Cycling probe detection has also been used in combination with other PCR and isothermal assays (See, e.g., US 2013/0203057 A1), and improvements to various aspects of the cycling probe reaction have enabled it to become a faster and more specific detection method.

The method described herein is specifically designed to allow the detection of nucleic acid targets, for example in a sample, through the combination of loop mediated isothermal amplification (LAMP) and

cycling probe detection (CP). The LAMP method is characterized by generating unique stem-loop structures, which contain single-stranded regions. These single-stranded regions provide ideal positions for single strand probe hybridization without the need to separate the double-stranded DNA either through heating or strand displacement enzymes. LAMP is performed isothermally and probe hybridization has been optimized to be carried out at the same temperature. Both of these enable the LAMP reaction and probe hybridization to occur simultaneously, thus greatly facilitating real-time probe-mediated detection and improving the detection speed. Therefore, in the methods described herein hybridization probes targeting sequences in the single-stranded loop regions were designed. To further improve the speed and sensitivity, the cycling probe technology is used for the hybridization probe design. In contrast to other probes that only generate one unit of signal per template, cycling probe could generate multiple units of signal per template through cycles of dissociating of digested probe and associating of intact probe on the same template. Moreover, compared with intercalating dyes (such as, but not limited to SYBR Green and related cyanine dyes, DAPI (4',6-diamidino-2-phenylindole), 7-AAD (7-aminoactinomycin D), Hoechst stains, Propidium iodide and EvaGreen) detection, which recognizes all double-stranded DNA, the cycling probe method only generates signal when the specific amplicon is produced, thus greatly increasing the assay specificity. The combination of LAMP and cycling probe detection, allows amplification of both the template and the probe signal simultaneously, thus achieving increased specificity and speed and also providing a multiplex possibility for isothermal nucleic acid detection. While there are various embodiments of how the LAMP method may be combined with the cycling probe method, two exemplary embodiments of such a combination are illustrated in Figure 1.

In a first aspect, the invention pertains to a method for determining the presence or amount of a target nucleic acid molecule in a sample by loop mediated isothermal amplification (LAMP). The method comprises:

- (a) combining (A) a LAMP reaction mixture, (B) a DNA polymerase with strand displacement properties, (C) at least one detection probe, (D) at least one cleaving agent, and (E) a sample containing the target nucleic acid molecule.
- (b) amplifying the target nucleic acid molecule by LAMP under suitable assay conditions that allow generation of the target amplicons by the LAMP reaction and multiple cycles of: hybridization of the at least one detection probe to the amplicons formed by the LAMP reaction, cleavage of the detection probe in the formed probe:target complex, and release of the cleaved probe fragments from the target ; and
- (c) detecting and optionally quantifying the released fragments of said at least one detection probe, and therefrom determining the presence or amount of the target nucleic acid molecule in the sample.

As used herein, the terms "LAMP" or "loop mediated isothermal amplification", refer to an isothermal amplification method, i.e. a method that is performed at an essential constant temperature without the need for a thermocycler. In LAMP, the target sequence is typically amplified at 60 to 65 °C using either

two or three sets of primers (i.e. 4 to 6 primers) and a polymerase with high strand displacement activity in addition to a replication activity. DNA polymerase with strand displacement activity/properties is known to those skilled in the art as an ability of the polymerase to displace the downstream DNA strand encountered during synthesis along the target strand. Typically, 4 different primers are used to identify 6 distinct regions on the target gene, which adds highly to the specificity (Figure 7). An additional "loop primer" or pair of "loop primers" can further accelerate the reaction. Due to the specific nature of the action of these primers, the amount of DNA produced in LAMP is considerably higher than PCR based amplification. The LAMP method is described in US Patent Nos. 6,410,278 B1 and 7,374,913 B2. Generally, the method uses two inner primers (forward inner primer = FIP and backward inner primer = BIP), two outer primers (F3 and B3), and optionally one or two, preferably two, loop primers (loop forward = LF and/or loop backward = LB). If two loop primers are used, one is preferably a loop forward primer and the other a loop backward primer. The inner primers comprise a target complementary region (typically referred to as F2 and B2) that facilitates hybridization and 5' thereto a sequence that is identical to a sequence in the target nucleic acid located upstream (5') relative to the sequence of the target bound by the target complementary region of the inner primer (typically referred to as F1c and B1c). Elongation of the inner primer by the polymerase thus creates a sequence comprising regions of self-complementarity in that the target-identical sequence on the 5' end of the inner primer (B1c) can, after elongation, bind to the synthesized sequence downstream of the target-complementary region of the inner primer (referred to as B1) and act as a primer for further extension. The outer primers bind to a target region in the target nucleic acid that lies downstream (i.e. 3') to the target region bound by the inner primers (referred to as F3c and B3c) and thus are responsible for the displacement of the elongated inner primer sequences from the template strand. The elongated inner primers are recognized and hybridized by the other primer of the inner primer pair and thus the dumbbell structured starting amplicons are generated. The dumbbell structures are then used for the following amplification, with the amplicons taking the form of concatemers. The principles of LAMP are for example disclosed by Eiken Chemical Co., Ltd. at <http://loopamp.eiken.co.jp/e/lamp/principle.html> and <http://loopamp.eiken.co.jp/e/lamp/loop.html>, in the publications of Nagamine et al. (Mol. Cell. Probes (2002) 16:223-229) and Notomi et al. (Nucleic Acids Res. (2000), 28 (12): e63) and the architecture of LAMP targets and primers is also schematically shown in Figure 7.

The target DNA sequence stretches can thus be schematically shown as:

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5'-F3c----F2c----F1c-----B1----B2----B3-3'
3'-F3-----F2-----F1-----B1c---B2c--B3c-5'

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The inner primers comprise the sequence elements 5'-F1c-F2-3' and 5'-B1c-B2-3'

The outer primers comprise the sequence elements 5'-F3-3' and 5'-B3-3'

The loop primer(s) typically target(s) a sequence in the loops between F2 and F1 and/or B2 and B1.

The principle of LAMP amplification is also schematically shown in Parida et al. (Rev. Med. Virol. (2008) 18:407-21) and is common general knowledge for those skilled in the art.

The term "target", as used herein, refers to the target nucleic acid to be detected but further encompasses the amplicons and concatemers produced by the LAMP reaction that include sequences of the target that are recognized by the inner primers, the loop primer(s) and/or the detection probes. Accordingly, when reference is made to a target that is bound by the LAMP primers or the detection probes, this term typically relates to the amplicons and concatemers as produced in the LAMP reaction, as these are more prevalent than the original target nucleic acid. "Amplicons" or "concatemers", as used interchangeably herein, relate to the amplified products generated starting from the template, i.e. the original target nucleic acid, and dumbbell starting structure produced from the inner primers in a first part of the LAMP reaction. These structures contain multiple repeats of the relevant sequence elements described above.

The sample may be any suitable sample and includes environmental samples, such as soil or water samples, as well as biological samples, such as tissue or biological fluids, including blood, plasma, serum, saliva and the like. The sample may be derived from a subject, suffering from or suspected of suffering from a disease, for example an infectious disease, the subject preferably being a mammal, for example a human. Alternatively, the subject may also be an animal or plant. If the method is used for pathogen detection, any sample type useful and known for such purpose may be used.

In accordance with the established principles of LAMP, the LAMP reaction mixture as used in the methods of the invention comprises a LAMP primer set of 4 to 6 primers, comprising two inner primers (FIP and BIP), two outer primers (F3 and B3), and optionally one or two loop primers (LF and/or LB). While it is known that the loop primer(s) increase(s) amplification efficiency, these are optional and not essential for carrying out the LAMP method. It is however preferred that one or two, preferably two, loop primers are included in the methods of the invention.

"At least one", as used herein, means one or more, for example 2, 3, 4, 5, 6, 7, 8, 9 or more. If used in relation to a component or agent, the term does not relate to the total number of molecules of the respective component or agent but rather to the number of different species of said component or agent that fall within the definition of broader term.

The two inner primers used in the methods thus each comprise a target complementary region on their 3' end (F2 and B2) and a target identical region on their 5' end (F1c and B1c), where in the target nucleic acid the sequence recognized by the target complementary region of the inner primers (termed F2c or B2c) lies 3' to the sequence identical to the target identical sequence on the 5' end of the inner primers (said sequence in the target termed F1c and B1c).

Accordingly, the two outer primers each comprise a target complementary region (F3 and B3), wherein in the target nucleic acid the sequence targeted by the target complementary region of the outer primers (termed F3c and B3c) are located 3' to the sequence of the target nucleic acid targeted by the target complementary region of the inner primers. This allows displacement of the elongated inner primers necessary for generation of the dumbbell shaped starting structures needed for concatemer formation in the later stages of LAMP.

The one or two optional loop primers each comprise a target complementary region that recognizes a sequence between the target complementary region on the 3' end of the inner primers or the complement thereof (i.e. the F2 or B2 region) and the sequence complementary to the target identical sequence on the 5' end of the inner primers or the complement thereof (i.e. the F1 or B1 region). The forward loop primers preferably bind between F1 and F2. Similarly, preferred binding for the backward loop primers is thus between B1 and B2. It may be preferred that the loop primer set comprises loop primers that bind between the F1 and F2 and loop primers that bind between the B1 and B2 regions of the amplicons.

The at least one detection probe is a single stranded probe comprising a DNA sequence complementary to the target nucleic acid, more particularly a region of the target nucleic acid that is amplified such that it is located in a loop region of the amplicons formed by the LAMP reaction. As described above, the loop regions are typically those regions that are located between the regions of self-complementarity and the target-complementary regions of the inner primer or the complements thereof (F2 and B2).

The probe is further designed such that it can hybridize to the amplicons/ concatemers formed under LAMP assay conditions to form a double-stranded probe:target complex. The hybridization is typically achieved by designing the probe sequence such that the nucleotides contained therein can form Watson-Crick base pairs with the designated target sequence in the amplicons/ concatemers. Generally, when reference is made herein to "complementarity", it is meant that the respective sequence can form Watson-Crick base pairs with its designated target or counterpart. "Fully complementary", which is generally desired for the targeting regions of the probes or the primers, thus means that the respective sequence stretch is complementary over the entire length of the respective region, i.e. each of the bases in the nucleotide sequence forms Watson-Crick base pairs with its counterpart sequence. Similarly, "complement", as used herein, relates to the fully complementary sequence of a given sequence, i.e. it forms a double-stranded molecule in that all nucleotides form Watson-Crick base pairs with the nucleotides in the opposite strand.

The at least one detection probe comprises a scissile linkage in the DNA sequence complementary to a (part of a) loop region of the amplicons formed by the LAMP reaction. The scissile linkage is preferably not naturally occurring in intact DNA molecules in that it is introduced artificially by chemical synthesis or similar methods and thus specifically recognizable by a suitable cleaving agent. The

scissile linkage is preferably located within the probe sequence such that adjacent to it there are target-complementary regions that can form Watson-Crick base pairs with the target sequence in the LAMP amplicons/ concatemers. "Adjacent" in this context preferably means that in 5' as well as in 3' direction relative to the scissile linkage there are sequence elements that base-pair with the target sequence. These sequence elements may have a length of at least 2 nucleotides, preferably of at least 3, at least 5, at least 7 or at least 10 nucleotides. As it is essential that under LAMP assay conditions the probe fragments are released from the target once the scissile linkage has been cleaved, the target-complementary sequence stretches in the probe adjacent to the scissile linkage may be designed such that the melting temperature of the respective probe fragment:target hybrids is below the temperature of the LAMP assay so that release is effected once the probe has been cleaved. The melting temperature of the probe is however selected such that the formed probe:target hybrid is stable under LAMP assay conditions as long as the probe is not cleaved.

The scissile linkage is selected such that it is cleavable in the probe:target complex but not the non-hybridized probe (under LAMP assay conditions). This may mean that the scissile linkage is a structural element only recognized and cleaved by the respective cleaving agent when it is present in a double-stranded structure, such as the formed probe:target complex. Examples for such scissile linkages will be discussed below, but include, without limitation, defects that may even occur naturally in the DNA and for which a repair mechanism exists that includes cleavage of the defective but not the intact strand. Concrete examples include one or more abasic nucleotides in a DNA sequence and/or RNA nucleotides in a DNA sequence, i.e. nucleotides that include ribose instead of deoxyribose.

In various embodiments, the at least one detection probe thus has the structure

DNA1-R-DNA2,

wherein DNA1 and DNA2 are DNA sequences that are complementary to adjacent regions in the target nucleic acid molecule and R is the scissile linkage. In various embodiments, it may be preferred that the whole probe sequence hybridizes to the target region in the amplicon/concatemer. In these embodiments, DNA1 and DNA2 both and optionally also R, depending on the nature of the linkage, hybridize to a continuous region in the target sequence. As also described above, it is preferred that the DNA sequences flanking the scissile linkage are of a length that is insufficient to maintain hybridized to the target under LAMP assay conditions once the probe has been cleaved. Depending on the actual sequence and the temperature used for the LAMP reaction, the flanking DNA sequences, i.e. DNA1 and/or DNA2, thus may be 3 to 25 nucleotides in length, typically 5 to 15 nucleotides in length.

In various embodiments, the detection probes of the invention range in length from about 10 nucleotides to about 50 nucleotides, preferably about 12 to 30 nucleotides. "About", as used herein, in relation to a numerical value means said value $\pm 10\%$, preferably $\pm 5\%$.

As described above, in various embodiments, the scissile linkage comprises at least one ribonucleotide with or without modifications. In some embodiments, it may be a single ribonucleotide flanked by DNA nucleotides, in other embodiments it may comprise 2 or more continuous ribonucleotides that together form the scissile linkage. It is preferred that the ribonucleotides are also complementary to the target sequence so that the full sequence of the DNA/RNA detection probe can hybridize to the target, preferably being fully complementary thereto. In such embodiments, where the scissile linkage comprises or consists of ribonucleotides, the cleaving agent comprises an agent that can selectively cleave the probe at the ribonucleotides. This may be an RNA cleaving enzyme, in particular a (thermostable) enzyme that recognizes DNA-RNA hybrids and selectively cleaves the RNA strand, such as RNase enzymes, including but not limited to RNase H, such as, e.g., RNase H2.

One embodiment to design the at least one detection probe with a scissile linkage is to utilize one or two adjacent 2'-fluoro-modified (ribo)nucleotides as RNase H2 recognition site. RNase H2 cleaves between the two 2'-fluoro-modified nucleotides when the detection probe forms a duplex with its complementary strand. RNase H2 cleavage activity is enhanced when two adjacent 2'-fluoro-modified nucleotides are employed instead of a single 2'-fluoro-modified nucleotide. Compared with the traditional cycling probe, which contains a single ribonucleotide as the recognition site, the cycling probe with two 2'-fluoro-modified nucleotides is more stable under heat and alkaline conditions when it is in single strand format, but is cleaved at a speed slower than that containing one ribonucleotide.

Alternatively, the scissile linkage comprises at least one apurinic/apyrimidinic site (AP site). In such embodiments, the respective AP site may form an unpaired position in the otherwise fully base-paired probe sequence or may form a loop that connects two adjacent paired nucleotides in the DNA sequence of the probe. In various embodiments, the cleaving agent comprises an agent that can selectively cleave the probe at the AP site. This may be an enzyme that recognizes AP sites in double-stranded nucleic acid structures and cleaves the strand containing the abasic site at the abasic site. In various such embodiments, the cleaving agent comprises or consist of an AP endonuclease, preferably with thermostable properties. Alternatively, the cleaving agent comprises a chemical agent (such as amine or purine derivatives), polyamine (such as spermine, spermidine), or nucleophilic peptide.

Alternatively, the scissile linkage comprises at least one modified deoxyribonucleotide. In such embodiments, the base of the deoxyribonucleotide may be methylated or substituted and the cleaving agent comprises a specific endonuclease that recognizes the base modification.

Generally, the at least one cleaving agent is (under LAMP assay conditions) capable of cleaving the at least one detection probe at said scissile linkage when said probe is hybridized to its target. Upon cleavage of said at least one detection probe at the scissile linkage the fragments of said at least one detection probe are released from the probe:target complex. This release makes the binding site on the target accessible for intact probes so that the cycle of detection probe binding, cleavage of the

probe and release of the probe fragments can be repeated. This results in signal amplification in that one template causes the generation of multiple signals by repeated cycles of probe binding, cleavage and release.

- 5 The cleaving agent is a chemical agent or an enzyme that specifically recognizes the double-stranded hybrids formed by the probe once bound to its target. In preferred embodiments, it is an enzyme that specifically recognizes the scissile linkage in the hybrid formed and cleaves the probe strand, but not the target strand. It is generally a preferred feature of the cleaving agent that it cleaves the target-bound but not the free probe and that cleavage is limited to the probe and does not affect the paired
10 target strand.

The cleavage typically occurs at a phosphodiester bond in the probe backbone close to or directly at the scissile linkage that is recognized by the cleaving agent. The cleavage leads to the generation of two probe fragments that due to the lowered affinity for the target, in particular a lowered melting
15 temperature of the probe fragment:target complex, cannot stay hybridized to the target under the elevated temperatures used for the LAMP assay.

The released fragments of the probe, with multiple fragments generated from each target molecule due to the cycles of probe binding, cleavage and release, as described above, can be determined by
20 any suitable means. Typically, they are detected and quantified.

While the fragments could in principle be detected by screening for the presence and amount of these short fragments, for example by gel electrophoresis, in various embodiments, the probe comprises a detectable marker that remains bound to one of the fragments of the probe after cleavage and can be
25 detected in the inventive methods.

In various embodiments, it can be preferred that the at least one detection probe comprises a detectable marker in form of a pair of moieties that provide a different signal when present in an intact probe compared to the cleaved probe fragments. In some embodiments, suitable markers comprise a
30 fluorescent marker. In some embodiments, these markers may comprise a fluorescence resonance energy transfer (FRET) pair consisting of two fluorophores, two chromophores or a fluorophore/chromophore quencher pair. "Fluorophores" emit fluorescence when excited by light of the proper wavelength. "Chromophores" are moieties that absorb a certain wavelength of visible light and thus appear colored. A "quencher" is a moiety that suppresses the fluorescence emission of a
35 fluorophore or the absorbance of a chromophore. In a FRET pair the two members of the pair influence each other as long as they are in close spatial proximity, for example when bound to the same molecule, with this influence becoming less pronounced the farther apart the two members are. This allows to detect the difference between the intact probe, where both moieties are in close proximity, and the cleaved probe, where each fragment comprises one member of the pair so that they
40 are no longer in close proximity to each other. In a typically fluorophore-quencher pair, the quencher

suppresses fluorescence of the fluorophore if both are present in the same molecule. Once both get separated by cleavage of the molecule such that both are no longer present in the same molecule, the influence of the quencher is reduced so that the fluorescence of the fluorophore is detectably increased.

5

In the detection probes, the first member of the pair may be located on the 5' end of the probe or in the sequence located 5' relative to the scissile linkage and the second member of the pair is located on the 3' end of the probe or in the sequence located 3' relative to the scissile linkage. The members of the pair are located such that they can interact in the intact non-cleaved probe and selected such that
10 the fluorescence signal changes upon cleavage of the probe. In the detection probes of the length described herein, this is typically given, even if both are located on the 5' and 3' end, respectively, of the probe.

These pairs are preferably bound to the probe such that upon cleavage each member remains on a
15 fragment different from that fragment bearing the other member. This ensures that the probe cleavage can be easily monitored.

Besides fluorescence, in various alternative embodiments lateral flow can also be used to detect cleavage of cycling probes. In such embodiments, the detection probe can be labelled on both ends
20 with markers that are recognized by antibodies. Examples of such markers include, without limitation, antigens including fluorescent markers that simultaneously function as antigen. Concrete examples include, without limitation, biotin, FITC and digoxigenin. As the probe is to be cleaved at the scissile linkage once it is bound to its target, in various embodiments the probe comprises two such markers on opposite ends of the probe, such that both are present in the intact probe but are present on
25 separate molecules after cleavage. In various embodiments, one of these markers may be FAM and the other may be biotin or digoxigenin. In a multiplexing method, the different probes for different targets may be labeled at one end (either 5' or 3' end) by one common marker, such as FAM, and by one marker in which they differ, such as biotin and digoxigenin, at the opposite end. In the lateral flow detection, the sample is typically run on a capillary bed after being put on a first element of the lateral
30 flow strip, the so-called sample pad. It then migrates to the second element, typically the conjugate pad wherein are typically stored the so-called detection conjugates, for example in a dried format together with a matrix that allows the binding reaction between the target molecule (e.g., an antigen) and its chemical partner (e.g., antibody) that has been immobilized. As the sample fluid dissolves the conjugates and the matrix, the sample and conjugate mix while flowing through the porous structure.
35 In this way, the analyte binds to the detection conjugates while migrating further through the capillary bed. This material has one or more areas (often called stripes) where a third or further "capture" molecule has been immobilized. By the time the sample-conjugate mix reaches these stripes, analyte has been bound by the detection conjugates and the "capture" molecule binds the complex. After a while, when more and more fluid has passed the stripes, complexes accumulate and the stripe-area
40 changes color. Typically, there are at least two stripes: one (the control) that captures any detection

conjugate and thereby shows that reaction conditions and technology worked fine and one that contains a specific capture molecule and only captures those conjugates which are complexed with an analyte molecule. After passing these reaction zones, the fluid enters the final porous material, the wick, that simply acts as a waste container.

5

In some embodiments, lateral flow detection includes the use of lateral flow strips that have at least 2 test bands that change color when the test sample contains the corresponding markers. In various embodiments, the color change is usually due to the use of a color marker (such as gold nanoparticle) conjugated with antibodies recognizing the markers in the test sample. For instance, the probe can be
 10 labelled with biotin and FITC on each end and the lateral flow strip contains gold nanoparticles conjugated with anti-FITC antibodies. Two separate test bands can be designed on the lateral flow strip such that one will turn color due to capture of complexes of gold nanoparticles and a cleaved probe fragment labelled with FITC (for example by detecting the anti-FITC antibody) and the other will turn color due to capture of complexes of gold nanoparticles and intact probes with both biotin and
 15 FITC (for example by binding biotin). In both cases, the detection signal would be caused by the gold nanoparticles conjugated to the anti-FITC antibodies, with the distinction made possible by immobilizing the respective capture agents on different parts of the strip (such as a capture agent for biotin, e.g. streptavidin located on a first location on the strip and a capture agent for the antibody, such as protein A, located on a second location of the strip). Regarding the location of test bands on
 20 the lateral flow strip, in this particular example it is important that the test band for detection of the intact probe should be placed upstream (i.e. in flow direction before) relative to that for detection of the cleaved probe (such that the sample first passes over the band for detection of the intact probe). By comparing with a control lateral flow strip that is tested only with intact probes, the test bands for cleaved probe and intact probe will be a darker color and lighter color than the corresponding test
 25 bands on control strip, respectively. This is due to the fact that in case the probe is mostly intact, it will nearly exclusively bind to the first location on the strip. However, in case the probe is cleaved, the part bound by the first capture agent (e.g. biotin) is no longer connected to the second marker on the probe which is bound by the antibody. In this case, the antibody bound to the other probe fragment passes over the first capture agent without substantial binding and is only bound at the second location by the
 30 second capture agent. Hence lateral flow strips can be used for detection of cleaved and/or intact probes depending on lateral flow strip design. Multiplex detection can also be accomplished with the use of multiple markers and test bands. These test bands may then be arranged in different locations on the lateral flow strips. The capture agents are typically immobilized in form of stripes that are arranged perpendicular to the flow direction. They may be arranged such that they form a barcoded
 35 pattern. Depending on the type of detection label used, the detection can be possible by the naked eye but can also be automated, for example by using an automatic scanner or barcode reader.

In various embodiments, the sequence in the target nucleic acid molecule recognized by the target complementary region of the at least one detection probe is located between the target
 40 complementary region of the inner primers (F2 or B2) and the sequence complementary to the target

identical sequence of the inner primers (F1 or B1). This means that the probe preferably binds in the loop regions of the dumbbell structured amplicons or the respective concatemers, similar to the loop primers. Accordingly, the disclosure above with relation to the preferred binding site for the loop primers similarly applies to the detection probe. Binding in this region ensures that probe:target hybridization does not interfere with the ongoing amplification reaction mediated by the inner primers.

The binding site for the detection probe in the loop regions is preferably different from the binding site of the loop primers, more preferably non-overlapping with the loop primer binding sites. In various embodiments, it lies in the same loop region and between the same elements recognized or contained in the inner primers, but lies 5' or 3', preferably 3', to the sequence targeted by the target complementary region of the loop primers.

As described above, in various embodiments, the sequences recognized by the target complementary regions of the LAMP primers and the at least one detection probe are non-overlapping.

In various embodiments, the method uses more than one detection probe for a single target nucleic acid molecule. This means that the method of the invention employs at least two of the detection probes as described above. While these at least two detection probes bind to the same target nucleic acid, i.e. the amplicons/ concatemers, it is preferred that they bind to target regions in the single-stranded loop regions, with the targeted regions not being identical or overlapping. It may, for example, be preferred that one of the probes binds in the loop between the F1/F1c region and the other binds in the loop between the B1/B1c region. Using multiple detection probes for the same target can increase signal strength and thus facilitate easier detection. Accordingly, in such embodiments, it can be preferred that the different detection probes all contain the same detectable marker, as a distinction between the signal from the different probes is not necessary.

In various embodiments, the method is a multiplexing method and is for determining the presence or amount of two or more target nucleic acid molecules in a sample. Such a method, if intended to allow distinction between different targets, typically comprises LAMP primers that either allow amplification of multiple targets with the same primer set or separate primer sets for separate targets. In some embodiments, it is also possible that only some of the primers are suitable for amplification of two separate targets while others of the primers need to be specifically designed for each target. Irrespective of whether one LAMP primer set or more LAMP primers are used for (multiple) target amplification, the method may use one or more different detection probes for each target nucleic acid molecule to facilitate distinction. This may be achieved, without limitation, by using detectable markers on the separate probes that can be distinguished in the detection. It is also possible to use one probe for multiple related target nucleic acid molecules.

The target nucleic acid used as a template for the LAMP reaction may be any nucleic acid molecule. In various embodiments, the target nucleic acid molecule is a nucleic acid of a pathogen, optionally a

human pathogen, preferably a bacterial, fungal, parasite or viral nucleic acid molecule. It is understood that when the target nucleic acid is an RNA, a cDNA reverse transcript may be used. In such embodiments, the method may comprise a reverse transcription of the target RNA. In various embodiments, the target may be the cDNA of a bacterial, fungal, parasite or viral RNA, for example a cDNA of a dengue virus RNA, including serotype 1 and/or serotype 3 dengue virus cDNA.

In some embodiments, where the target is dengue virus cDNA,

- (1) the LAMP inner forward primer comprises the nucleic acid sequence set forth in SEQ ID NO:1, 2 or 3 (GB_T1_3UTR_FIP and GB_T1T3_3UTR_FIP and GB_T3_3UTR_FIP) or a variant thereof having at least 90 % sequence identity over the entire length;
- (2) the LAMP inner backward primer comprises the nucleic acid sequences set forth in SEQ ID NO: 4 (GB_T1_3UTR_BIP) or a variant thereof having at least 90 % sequence identity over the entire length;
- (3) the LAMP outer forward primer comprises the nucleic acid sequence set forth in SEQ ID NO:5 (GB_T1_3UTR_F3) or a variant thereof having at least 90 % sequence identity over the entire length;
- (4) the LAMP outer backward primer comprises the nucleic acid sequence set forth in SEQ ID NO:6 (GB_T1_3UTR_B3) or a variant thereof having at least 90 % sequence identity over the entire length;
- (5) the LAMP loop forward primer, if present, comprises the nucleic acid sequence set forth in SEQ ID NO:7 or 8 (GB_T1_3UTR_LPF or GB_T1T3_3UTR_LPF) or a variant thereof having at least 90 % sequence identity over the entire length; and
- (6) the LAMP loop backward primer, if present, comprises the nucleic acid sequence set forth in SEQ ID NO:9 (GB_T1_3UTR_LPB) or a variant thereof having at least 90 % sequence identity over the entire length.

The above primers allow the detection and quantification of dengue virus, such as dengue virus serotype 1 and 3, as described in greater detail in the examples. The primers of SEQ ID NO:1 and 3 may allow amplification of dengue virus serotype 1 and 3 cDNA, respectively, and the primer with SEQ ID NO:2 may allow amplification of both. Similarly, the primer with SEQ ID NO:7 may be designed for serotype 1 amplification and the primer with SEQ ID NO:8 for serotype 3 amplification.

In the above embodiments of the methods for dengue virus detection, the at least one detection probe may comprise the nucleic acid sequence set forth in any one of SEQ ID Nos. 10-12 (T1 3UTR F Probe, T3 3UTR F Probe, T1_3UTR_B2LP_CP_rA) or a variant thereof having at least 90 % sequence identity over the entire length. SEQ ID NO:10 and 12 may be for serotype 1 detection, while SEQ ID NO:11 may be for serotype 3 detection.

When reference is made to sequence identity, this means that in a given nucleic acid molecule the respective nucleotide at a given position is identical to the nucleotide in a reference nucleic acid

molecule at the corresponding position. The level of sequence identity is given in % and can be determined by an alignment of the query sequence with the template sequence.

The determination of the identity of nucleotide sequences is achieved by a sequence comparison. This comparison or alignment is based on the BLAST algorithm well-established and known in the art (See, e.g., Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990): "Basic local alignment search tool", J. Mol. Biol. 215:403-410, and Altschul, Stephan F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997): "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs"; Nucleic Acids Res., 25, S.3389-3402) and is in principle carried out by aligning stretches of nucleotides in the nucleotide sequences with each other. Another algorithm available in the art is the FASTA algorithm. Sequence comparisons (alignments), in particular multiple sequence comparisons, can be generated using computer programs. Commonly used are for example the Clustal series (See, e.g. Chenna et al. (2003): "Multiple sequence alignment with the Clustal series of programs," Nucleic Acid Research 31, 3497-3500), T-Coffee (See, e.g., Notredame et al. (2000): "T-Coffee: A novel method for multiple sequence alignments", J. Mol. Biol. 302, 205-217) or programs based thereon or the respective algorithms. Further possible are sequence comparisons (alignments) with the computer program Vector NTI® Suite 10.3 (Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA, USA) with the pre-set standard parameters, the AlignX-module of which is based on ClustalW. If not explicitly defined otherwise, sequence identity is determined using the BLAST algorithm.

Such a comparison allows determining the identity of two sequences and is typically expressed in % identity, i.e. the portion of identical nucleotides in the same or corresponding positions. If not explicitly stated otherwise, the sequence identities defined herein relate to the percentage over the entire length of the respective sequence, i.e. typically the reference sequence. If the reference sequence is 20 nucleotides in length, a sequence identity of 90 % means that 18 nucleotides in a query sequence are identical while 2 may differ.

In another aspect, the invention also relates to a kit for the determining of the presence or amount of a target nucleic acid molecule in a sample by loop mediated isothermal amplification (LAMP). The kit may be designed such that it allows performing the methods of the invention. Accordingly, the kit may comprise:

- A. a LAMP reaction mixture;
- B. a DNA polymerase with strand displacement properties;
- C. at least one detection probe under conditions that allow the LAMP reaction to occur; and
- D. at least one cleaving agent for the at least one detection probe.

The components A-D are, in various embodiments, defined as described above for the identical components in relation to the methods of the invention.

In various embodiments, the LAMP reaction mixture comprises a LAMP primer set of 4 to 6 primers, comprising two inner primers (FIP and BIP), two outer primers (F3 and B3), and optionally one or two loop primers (LF and/or LB), the primers being specific for the target nucleic acid molecule.

- 5 In various embodiments, the at least one detection probe is a single stranded probe comprising a DNA sequence complementary to a sequence in a loop region of the amplicons formed by the LAMP reaction that can hybridize to said amplicons under LAMP assay conditions and form a double-stranded probe:target complex, the at least one detection probe comprising a scissile linkage in the DNA sequence complementary to a sequence in a loop region of the amplicons formed by the LAMP
10 reaction, wherein said scissile linkage in the probe:target complex but not the non-hybridized probe is cleavable under LAMP assay conditions and said cleavage leads to release of one or more fragments of said at least one detection probe adjacent to said scissile linkage from the probe:target complex.

- In various embodiments, under LAMP assay conditions the at least one cleaving agent is capable of
15 cleaving the at least one detection probe at said scissile linkage when said probe is hybridized to its target, wherein upon cleavage of said at least one detection probe at the scissile linkage the fragments of said at least one detection probe are released from the probe:target complex.

The methods and kits described herein have a number of advantages over existing techniques:

- 20 1. Compared with existing LAMP and probe detection methods, the cycling probe detection method as described herein generates more than one unit of signal per amplicon, in contrast to only one unit of signal per amplicon from other LAMP with probe detection methods. Therefore, stronger signal, faster speed and higher sensitivity are possible.
- 25 2. Design of the detection probes is independent of other LAMP primers, rendering the assay design much more flexible.
3. The detection probe is recognizing and hybridizing to a sequence in a single-stranded loop region (which is not present in any primer sequence), which increases LAMP specificity dramatically by eliminating detection of non-specific primer dimer amplification.
- 30 4. The efforts for primer design, screening and optimization for LAMP reactions to reduce non-specific signals are greatly reduced /eliminated.
5. Multiplexing of LAMP becomes more straightforward.

Examples

Materials and Methods

RT-LAMP primers were used for both LAMP and RT-LAMP in the subsequent experiments. Modifications were made to the FIP primers used for multiplexing Dengue serotype 1 and 3. The sequences are shown in Table 1 below. Serotype-specific detection probes were designed to bind to a loop region of the LAMP amplicon and their sequences are shown in Table 2 below. Cy5 (CAS: 146368-14-1) and 6-FAM (6-carboxyfluorescein) are the fluorophores coupled to the 5' end of the probes. Quenchers were coupled to the 3' end, with IAbRQSp being 3' Iowa Black® RQ (Integrated DNA Technologies, Coralville, Iowa, USA (IDT)) and BHQ-1 being Black Hole Quencher® 1 (Biosearch Technologies Inc., Teddington, Middlesex, UK).

The 25 µL LAMP reaction contained 2.46 µM of FIP and BIP primers (inner primers), 0.62 to 1.23 µM of LPF and LPB primers (loop primers), and 0.31 µM of F3 and B3 primers (outer primers) in 1X ThermoPol Buffer or Isothermal Amplification Buffers (New England Biolabs, Ipswich, Mass, USA (NEB): B90045, B0537S, B0374S), with 8 mM of MgSO₄ (NEB; B10035), 100-250 mU of RNase H2 (IDT; 11-02-12-01), 8 U of *Bst* polymerase (NEB; M0275L, M0537L, or M0374L), 1.4 mM each of dNTPs (Bioline, Swedesboro, New Jersey, USA; BIO-39029), 0.2X of SYBR Green (Thermo Fisher Scientific, Grand Island, New York, USA; S7563) and 6x10¹¹⁻¹³ copies of Cy5/FAM labelled probes. 7.5 U of WarmStart RTx (NEB; M0380L) was added for each RT-LAMP reaction. DNA plasmids were heated at 98 °C for 3 mins and rapidly cooled on ice before being used for LAMP reactions.

The reactions were run in a Rotor-Gene Q (RGQ) cycler (QIAGEN, Germantown, Maryland, USA) at 60-65 °C for 60 cycles (1 min/cycle) with individual gain optimizations for SYBR Green (1-3FI) and Cy5/FAM (5-10FI). For RT-LAMP reactions, reverse transcription was performed at 65 °C for 5 mins in a thermomixer, followed by addition of *Bst* enzyme, RNase H2 and/or detection probe to the reactions that were then run in the RGQ cycler for the LAMP amplification.

Unless otherwise stated, ThermoPol buffer and *Bst* polymerase, Large Fragment polymerase (NEB) were used in the experiments.

Table 1: LAMP/RT-LAMP primer sequences.

Primers	Sequences (5' → 3')
GB_T1_3UTR_FIP	CCACCAGGGTACAGCTTCCCGACCCCTCCCAAAACACAA (SEQ ID NO:1)
GB_T1_3UTR_BIP	AGAGGTTAGAGGAGACCCCC- AGCAGGATCTCTGGTCTCTC (SEQ ID NO:4)
GB_T1_3UTR_LPF	TGGTGTGGGGCCCGCT (SEQ ID NO:7)
GB_T1_3UTR_LPB	ACAGCATATTGACGCTG (SEQ ID NO:9)
GB_T1_3UTR_F3	TGGGGTAGCAGACTAGTGG (SEQ ID NO:5)
GB_T1_3UTR_B3	TCTGTGCCTGGAATGATGC (SEQ ID NO:6)
GB_T1T3_3UTR_FIP	AGTCCTTACCACCAGGGTACGACCCCTCCCAAAACACAA (SEQ ID NO:2)
GB_T3_3UTR_FIP	AGTCCTTTGCAAGGAGGTACGACCCCTCCCAAAACACAA (SEQ ID NO:3)
GB_T1T3_3UTR_LPF	TTGGGCCCGCTGCTGCG (SEQ ID NO:8)

**DENV3 LAMP primers were modified from the DENV1 primers set to accommodate the multiplexing of DENV1 and DENV3.*

5 Table 2: Cycling probe sequences.

Probes	Sequence (5' → 3')
T1_3UTR_B2LP_CP_rA (8-1-15)	Cy5-CAACAATArAACAGCATATTGACGC- IAbRQSp (SEQ ID NO:12)
T1 3UTR F Probe	6-FAM-AGCTTCCCCrUGGTGTTGGGCC-BHQ-1 (SEQ ID NO:10)
T3 3UTR F Probe	Cy5-AGCTTCCCTrCAGTGCTCGGGCC-IAbRQSp (SEQ ID NO:11)

rA, rU and rC indicate that the respective nucleotide (A, U, C) is a ribonucleotide.

10 In a proof of concept assay, LAMP assays were performed using SYBR Green as the detection reagent (control) and fluorophore/quencher labelled detection probes. The results are shown in Figures 2A (control) and 2B. In the control reaction with SYBR Green, non-specific amplification signals were detected after 12 mins. In the method according to the invention, the time taken for Cy5 fluorescence to be detected was similar to that of SYBR Green but the non-specific amplification signals for the NTC (no target controls) were successfully suppressed.

15 The sensitivity of the inventive method was assessed and depicted in Fig. 3B. Again, a control with SYBR Green was carried out in parallel (Fig. 3A)

20 Figure 3A: SYBR Green signal for isothermal amplification at 65 °C. The LAMP method was able to amplify 100 and 500 copies of plasmid DNA template consistently. However, non-specific amplification signals were detected after 25 mins.

Figure 3B: Isothermal amplification at 65 °C of plasmid DNA detected via Cy5 fluorescence released by cycling probe cutting. LAMP amplification coupled with fluorescent probe cutting performed similarly

to SYBR Green intercalation. Additionally, the non-specific amplification signals for the NTC were successfully suppressed.

In another set of experiments, RT-LAMP was carried out. Again, the SYBR Green control is shown in Fig. 4A and the inventive method shown in Fig. 4B. In the control reaction, non-specific amplification signals were detected after 20 mins. In the inventive method, except for a slight delay, Cy5 signals were comparable to SYBR Green signals. Furthermore, the non-specific amplification signals for the NTC were suppressed.

In the next set of experiments, the multiplexing properties were assessed in proof-of-concept assays using 6-FAM and Cy5 as detection reagents coupled to the probe to allow distinction between serotype 1 and 3. The results for the FAM channel are shown in Fig.5A, with the results for the Cy5 channel shown in Fig. 5B. In the reaction monitoring the FAM channel (DENV1 RNA-specific), only the signal from DENV1 RNA reaction was observed at around 15 min of the amplification while the DENV3 RNA reaction did not show any significant fluorescence signal. There were no signals observed from NTC reactions. In the reaction monitoring the Cy5 channel (DENV3-specific), only the signal from DENV3 RNA reaction was observed at around 10 min of the amplification while the DENV1 RNA reaction did not show any significant signal. There were no signals observed from NTC reactions.

In another set of experiments, the advantage of the cleavable cycling probes over simple binding of uncleavable probes was assessed. Reactions comprised probes labeled with Cy5 (Fig.6A, high NEC signal) or FAM (Fig. 6B, low NEC signal) in the presence or absence of cleaving enzyme, RNase H2 (no enzyme control, NEC). NTC means no target control. In the presence of RNase H2, probe cutting resulted in much higher Cy5 fluorescence compared to probe binding alone (NEC). In the case of FAM-labeled probes, probe binding alone did not result in fluorescence signals that can be readily differentiated from NTC background.

1
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<120> NUCLEIC ACID DETECTION METHOD USING LAMP AND PROBE DETECTION

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Claims

1. Method for determining the presence or amount of a target nucleic acid molecule in a sample using loop mediated isothermal amplification (LAMP), the method comprising:

- (a) combining a LAMP reaction mixture, a DNA polymerase with strand displacement properties, a sample containing the target nucleic acid molecule, at least one detection probe, and at least one cleaving agent;
 wherein the LAMP reaction mixture comprises a LAMP primer set of 4 to 6 primers, comprising two inner primers (FIP and BIP), two outer primers (F3 and B3), and optionally one or two loop primers (LF and/or LB); and
 wherein the at least one detection probe is a single stranded probe comprising a DNA sequence complementary to a sequence in a loop region of the amplicons formed by the LAMP reaction that can hybridize to said amplicons under LAMP assay conditions and form a double-stranded probe:target complex, the at least one detection probe comprising a scissile linkage in the DNA sequence complementary to a sequence in a loop region of the amplicons formed by the LAMP reaction, wherein said scissile linkage in the probe:target complex but not the non-hybridized probe is cleavable under LAMP assay conditions;
 wherein under LAMP assay conditions the at least one cleaving agent is capable of cleaving the at least one detection probe at said scissile linkage when said probe is hybridized to its target, wherein upon cleavage of said at least one detection probe at the scissile linkage the fragments of said at least one detection probe are released from the probe:target complex so that the cycle of detection probe binding, cleavage of the probe and release of the probe fragments can be repeated;
- (b) amplifying the target nucleic acid molecule by LAMP under suitable assay conditions that allow generation of the target amplicons by the LAMP reaction and multiple cycles of: (i) hybridization of the at least one detection probe to the amplicons formed by the LAMP reaction, (ii) cleavage of the detection probe in the formed probe:target complex, and (iii) release of the cleaved probe fragments from the target; and
- (c) detecting and optionally quantifying the released fragments of said at least one detection probe, and therefrom determining the presence or amount of the target nucleic acid molecule in the sample.

2. The method of claim 1, wherein

- (1) the two inner primers each comprise a target complementary region on their 3' end (F2 and B2) and a target identical region on their 5' end (F1c and B1c), wherein in the target nucleic acid the sequence targeted by the target complementary region of the inner primers lies 3' to the sequence identical to the target identical sequence on the 5' end of the inner primers; and
- (2) the two outer primers each comprise a target complementary region (F3 and B3), wherein in the target nucleic acid the sequence targeted by the target complementary region of

the outer primers is located 3' to the sequence of the target nucleic acid targeted by the target complementary region of the inner primers; and, optionally,

- (3) the one or two loop primers each comprise a target complementary region that targets a sequence of the amplicons formed between the target complementary region on the 3' end of the inner primers and the sequence complementary to the target identical sequence on the 5' end of the inner primers.

3. The method of claim 1 or 2, wherein the at least one detection probe has the structure DNA1-R-DNA2,

wherein DNA1 and DNA2 are DNA sequences that are complementary to adjacent regions in the target nucleic acid molecule and R is the scissile linkage.

4. The method of any one of claims 1 to 3, wherein the scissile linkage comprises at least one ribonucleotide or modified ribonucleotide and the cleaving agent comprises a thermostable RNase H.

5. The method of any one of claims 1 to 4, wherein the scissile linkage comprises at least one apurinic/apyrimidinic site (AP site) and the cleaving agent comprises an enzyme, preferably a thermostable AP endonuclease, a chemical compound, preferably an amine or purine derivative, a polyamine, or a nucleophilic peptide.

6. The method of any one of claims 1 to 4, wherein the scissile linkage comprises at least one deoxyribonucleotide with its base methylated or substituted and the cleaving agent comprises a specific endonuclease that recognizes the base modification.

7. The method of any one of claims 1 to 6, wherein the released fragments are labeled with a detectable marker and labeled fragments are detected in step (c).

8. The method of any one of claims 1 to 6, wherein the at least one detection probe comprises a fluorescent marker, preferably a fluorescence resonance energy transfer (FRET) pair consisting of two fluorophores, two chromophores or a fluorophore/chromophore quencher pair, wherein the first member of the pair is located on the 5' end of the probe or in the sequence 5' to the scissile linkage and the second member of the pair is located on the 3' end of the probe or in the sequence 3' to the scissile linkage, wherein the members of the pair are located such that they can interact in the intact non-cleaved probe and selected such that the fluorescence signal changes upon cleavage of the probe.

9. The method of any one of claims 1 to 6, wherein the detection method in step (c) is lateral flow detection.

10. The method of any one of claims 1 to 9, wherein the sequence in the target nucleic acid molecule targeted by the target complementary region of the at least one detection probe is located between the sequence targeted by the target complementary region on the 3' end of the inner primers and the sequence identical to the target identical sequence on the 5' end of the inner primers and, optionally, 5' or 3', preferably 3', to the sequence in the target nucleic acid molecule targeted by the target complementary region of the loop primer(s).
11. The method of any one of claims 1 to 10, wherein the sequences targeted by the target complementary regions of the LAMP primers and the at least one detection probe are non-overlapping.
12. The method of any one of claims 1 to 11, wherein the method uses more than one detection probe for a single target nucleic acid molecule.
13. The method of any one of claims 1 to 12, wherein the method is a multiplexing method and is for determining the presence or amount of two or more target nucleic acid molecules in a sample, wherein the method uses one or more LAMP primer sets and/or one or more detection probes for each target nucleic acid molecule or for multiple related target nucleic acid molecules.
14. The method of any one of claims 1 to 13, wherein the target nucleic acid molecule is a nucleic acid of a pathogen, optionally a human pathogen, preferably a bacterial, fungal, parasite or viral nucleic acid molecule or a cDNA reverse transcript of a bacterial, fungal, parasite or viral RNA, preferably a cDNA of a dengue virus RNA.
15. The method of claim 14, wherein
 - (1) the LAMP inner forward primer comprises the nucleic acid sequence set forth in SEQ ID NO:1, 2 or 3 (GB_T1_3UTR_FIP and GB_T1T3_3UTR_FIP and GB_T3_3UTR_FIP) or a variant thereof having at least 90 % sequence identity over the entire length;
 - (2) the LAMP inner backward primer comprises the nucleic acid sequences set forth in SEQ ID NO: 4 (GB_T1_3UTR_BIP) or a variant thereof having at least 90 % sequence identity over the entire length;
 - (3) the LAMP outer forward primer comprises the nucleic acid sequence set forth in SEQ ID NO:5 (GB_T1_3UTR_F3) or a variant thereof having at least 90 % sequence identity over the entire length;
 - (4) the LAMP outer backward primer comprises the nucleic acid sequence set forth in SEQ ID NO:6 (GB_T1_3UTR_B3) or a variant thereof having at least 90 % sequence identity over the entire length;
 - (5) the LAMP loop forward primer, if present, comprises the nucleic acid sequence set forth in SEQ ID NO:7 or 8 (GB_T1_3UTR_LPF or GB_T1T3_3UTR_LPF) or a variant thereof having at least 90 % sequence identity over the entire length; and

(6) the LAMP loop backward primer, if present, comprises the nucleic acid sequence set forth in SEQ ID NO:9 (GB_T1_3UTR_LPB) or a variant thereof having at least 90 % sequence identity over the entire length.

- 5 16. The method of claim 14 or 15, wherein the at least one detection probe comprises the nucleic acid sequence set forth in any one of SEQ ID Nos. 10-12 (T1 3UTR F Probe, T3 3UTR F Probe, T1_3UTR_B2LP_CP_rA) or a variant thereof having at least 90 % sequence identity over the entire length.
- 10 17. Kit for the determining of the presence or amount of a target nucleic acid molecule in a sample by loop mediated isothermal amplification (LAMP), the kit comprising:
- 15 A. a LAMP reaction mixture;
- B. a DNA polymerase with strand displacement properties;
- C. at least one detection probe under conditions that allow the LAMP reaction to occur; and
- D. at least one cleaving agent for the at least one detection probe;
- wherein the LAMP reaction mixture comprises a LAMP primer set of 4 to 6 primers, comprising two inner primers (FIP and BIP), two outer primers (F3 and B3), and optionally one or two loop primers (LF and/or LB), the primers being specific for the target nucleic acid molecule;
- 20 wherein the at least one detection probe is a single stranded probe comprising a DNA sequence complementary to a sequence in a loop region of the amplicons formed by the LAMP reaction that can hybridize to said amplicons under LAMP assay conditions and form a double-stranded probe:target complex, the at least one detection probe comprising a scissile linkage in the DNA sequence complementary to a sequence in a loop region of the amplicons formed by the LAMP reaction, wherein said scissile linkage in the probe:target complex but not the non-hybridized
- 25 probe is cleavable under LAMP assay conditions and said cleavage leads to release of one or more fragments of said at least one detection probe adjacent to said scissile linkage from the probe:target complex; and
- wherein under LAMP assay conditions the at least one cleaving agent is capable of cleaving the at least one detection probe at said scissile linkage when said probe is hybridized to its target,
- 30 wherein upon cleavage of said at least one detection probe at the scissile linkage the fragments of said at least one detection probe are released from the probe:target complex so that the cycle of probe binding, cleavage and release of the probe fragments can be repeated.