The disclosure relates to novel probes for use in LAMP detection methods. The probes contain a single fluorophore label bound to an internal cytosine residue of the probe. The probes are particularly useful in the detection of *chlamydia* and *gonorrhea* infections in a patient.
Schematic of MAST DNA probe

5' ACGTGAAC GCTTATACGCTC

3'

5'T GCA CT GCGAA TC TG CAG3'

FIG1
NUCLEIC ACID PROBE WITH SINGLE FLUOROPHORE LABEL BOUND TO INTERNAL CYTOSINE FOR USE IN LOOP MEDIATED ISOTHERMAL AMPLIFICATION

FIELD OF THE INVENTION

[0001] The present invention relates to a probe for the detection of a nucleic acid, a method using said probe and a kit of parts. Preferably the probe of the invention is useful in a method for the detection of nucleic acids derived from Chlamydia trachomatis and/or Neisseria gonorrhoeae and may be used in the diagnosis of Chlamydia and/or Gonorrhoea infections.

REFERENCE TO SEQUENCE LISTING

[0002] A Sequence Listing submitted as an ASCII text file via EFS-Web is hereby incorporated by reference in accordance with 35 U.S.C. §1.52(e). The name of the ASCII text file for the Sequence Listing is 23106975_1.TXT, the date of creation of the ASCII text file is Apr. 12, 2016, and the size of the ASCII text file is 17.3 KB.

BACKGROUND OF THE INVENTION

[0003] Nucleic acid amplification is one of the most valuable tools in the life sciences field, including application-oriented fields such as clinical medicine, in which diagnosis of infectious diseases, genetic disorders and genetic traits is particularly beneficial. In addition to the widely used PCR-based detection (Saiki R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. and Arnheim, N. (1985) Science, 230, 1350-1354), several amplification methods have been invented. Examples include nucleic acid sequence-based amplification (NASBA), self-sustained sequence replication (3SR) and loop-mediated isothermal amplification (LAMP). PCR uses heat denaturation of double-stranded DNA products to promote the next round of DNA synthesis. 3SR and NASBA eliminate heat denaturation by using a set of transcription and reverse transcription reactions to amplify the target sequence.

[0004] These methods can amplify target nucleic acids to a similar magnitude, all with a detection limit of less than 10 copies and within an hour or so. They require either a precision instrument for amplification or an elaborate method for detection of the amplified products due to poor specificity of target sequence selection. Despite the simplicity and the obtainable magnitude of amplification, the requirement for a high precision thermal cycler in PCR prevents this powerful method from being widely used, such as in private clinics as a routine diagnostic tool. In contrast, LAMP is a method that can amplify a few copies of DNA to over 100 in less than an hour under isothermal conditions and with greater specificity.

[0005] As with other molecular-probe based technologies identified above, loop-mediated isothermal amplification (LAMP) assays can be used to detect the presence of specific microorganisms in a sample. However, the detection methods are based on direct visual detection, turbidity or via a non-specific DNA intercalating dye. Direct visual measurement is end point measurement and is unable to provide real time analysis. Turbidity and non-specific intercalating dyes do provide real time analysis of amplification which occurs however this is non-specific i.e. all amplification is detected whether this is true positive amplification or false amplification due to mis-priming, cross specificity.

SUMMARY OF THE INVENTION

[0006] In accordance with a first aspect of the present invention there is provided a probe for isothermal nucleic acid amplification comprising an oligonucleotide probe sequence complementary to a region of a target nucleic acid sequence, wherein said oligonucleotide probe sequence has only one fluorophore ligand and which ligand is bound to an internal cytosine base and wherein said oligonucleotide probe sequence does not have a 3' end terminator.

[0007] In a preferred embodiment to oligonucleotide probe sequence is a DNA sequence and the target nucleic acid sequence is a DNA sequence.

[0008] Preferably, fluorescence increases to indicate the presence of the target nucleic acid in a sample.

[0009] The cytosine base is preferably substantially centrally disposed along the oligonucleotide's length. There are particular benefits associated with labeling the probe internally at a cytosine base. The specificity of the DNA product amplified in an isothermal reaction may be confirmed using a melt curve analysis. However due to a large number of product variants generated in this reaction and a low resolution of melt curve analysis, using intercalating dyes like V13, it is very difficult to distinguish between specific and unspecific DNA products generated under isothermal conditions. Commonly used probes such as TaqMan® probe are not compatible with LAMP technology due to the strand displacement activity of BST polymerase. The probe of the invention is elongated and becomes incorporated into a DNA product during isothermal amplification, which allows for performing a melt curve analysis on the generated product. In the probe of the invention, the fluorophore is conjugated to an internal cytosine complementary to guanine in the antisense strand. Guanine affects the excitation state of many fluorophores resulting in a formation of unique melt curve signatures and allows distinguishing between specific and unspecific products generated under isothermal conditions.

[0010] The oligonucleotide does not contain a dNTP at its 3' end which enables incorporation of the labelled oligonucleotide into the amplicon. Thus, the 3' end of the probe is not “blocked”.

[0011] The fluorophore may comprise any one or more selected from the following: FAM, JOE, TET, HEX, TAMRA, ROX, ALEXA and ATTO.

[0012] The probe may comprise the following sequence: 5' Xn C*Xm 3'

[0013] Where n is >1, m is >3, X is nucleotide base; and * is a fluorophore. Preferably, the nucleotide base is selected from A, T, C and G. Preferably, n is more than 1 to 20 or less, more preferably more than 1 to 10 or less. Preferably, m is more than 3 to 20 or less, more preferably more than 3 to 10 or less. It is contemplated that all combinations of lengths of probe covered by the possible number of nucleotides that n or m make take by the preceding ranges are disclosed.

[0014] Preferably, the probe may comprise a sequence selected from any one of the following sequences:

SEQ ID NO. 2:
TAAGATAACC-FAMCCGCACGTG (CT PB1-FAM internal)

SEQ ID NO. 4:
GCCGACRTA [C-ALEXA546] CAGCTATGATCAA (GC por7-joe loopF)
The fluorescence is preferably increased when the oligonucleotide is incorporated into the target nucleic acid sequence which results in a change in the configuration of the amplicon-probe complex leading to an alteration of the fluorophore excitation state.

The cytosine bound to the fluorophore ligand is not disposed at or proximate to the 5' or 3' end. More preferably it is not disposed in the first 3 bases from either the 5' or 3' end. Preferably the cytosine bound to the fluorophore is disposed at the middle base of the probe.

In accordance with a further aspect of the present invention, there is provided an isothermal nucleic acid amplification probe as described hereinabove.

In accordance with a further aspect of the present invention, there is provided a loop-mediated isothermal amplification probe as described above.

Methods and compositions for determining at least one target nucleic acid in a mixture of nucleic acids generally employ a probe, a hybridizing reagent, and one or more phosphate bond-forming enzymes associated with any required nucleotide triphosphates to form a nucleic acid chain.

These methods usually involve amplification, such as including the use of a promoter in conjunction with a DNA polymerase, a restriction site where only one strand is cleaved and is then displaced by extension with a DNA polymerase, or a circular hybridizing reagent, where concatenated repeats are produced. Detection of the amplified nucleic acid may take many forms but preferably via a fluorophore.

In accordance with a further aspect of the present invention, there is provided a method of detecting a target nucleic acid in a sample comprising:

a. amplifying a target nucleic acid in the sample to provide an amplified nucleic acid;

b. probing the amplified nucleic acid with a probe as described hereinabove; and

c. detecting the presence of a single or multiple target nucleic acids.

The target nucleic acid may be from a microorganism, fungi, yeast, virus, human, animal, plant etc. The target nucleic acid for LAMP is known to enable LAMP primers and appropriately specific probes to be synthesised. Thus, the presence or absence of said micro-organism, fungi, yeast, virus, human, animal or plant in a sample can be determined. Preferably the target nucleic acid is from *Chlamydia trachomatis* or *Neisseria gonorrhoeae*.

Preferably, fluorescence increases to indicate the presence of the target nucleic acid in a sample.

The process is isothermal, and allows for amplification in a single stage or sequential stages in a single vessel, where all of the reagents are compatible.

In a further aspect, the present invention provides a method of diagnosing *Chlamydia* and/or *Gonorrhea* in a patient, comprising

- providing a sample derived from the patient;
- adding one or more probes of the present invention to the sample; and
- detecting the presence of a nucleic acid derived from *Chlamydia trachomatis* and/or *Neisseria gonorrhoeae* wherein an increase in the fluorescence of the probe indicates the presence of a *Chlamydia trachomatis* and/or *Neisseria gonorrhoeae* infection.

The sample may be treated by routine methods to enable the probe to bind with any target nucleotide present in the sample. Such treatment may include centrifuging and lysing the sample to release any target nucleic from the infecting microorganism.

In one embodiment, a single type of probe specific for a nucleic acid from either *Chlamydia trachomatis* or *Neisseria gonorrhoeae* is used in the method such that either *Chlamydia trachomatis* or only *Neisseria gonorrhoeae* is detected in the sample.

In a preferred embodiment, at least two different probes are added to the sample wherein a first probe is labelled with a first fluorescent label and is specific for probing *Chlamydia trachomatis* nucleic acid and a second probe is labelled with a different fluorescent label to the first probe and is specific for probing *Neisseria gonorrhoeae* nucleic acid. In this embodiment, it is possible to simultaneously detect a *Chlamydia* and a *Gonorrhea* infection in a single sample derived from a patient.

In one aspect of the method of the invention, the sample from the patient may be a blood sample, urine sample, serum sample or saliva sample.

In accordance with a further aspect of the present invention there is provided a kit comprising a probe as described hereinabove, LAMP reaction buffer containing a polymerase enzyme, dNTPs and LAMP primers for the target.

In one embodiment a positive and negative control may be included in the kit. The reagents may be presented as wet reagents or in lyophilised form.

The buffer used in the method or kit of the invention comprises dNTPs at a concentration of from 1-10 mM, one or more salts at a concentration of from 2-20 mM, Tris pH 8.8 at a concentration of from 10-100 mM, Trehalose at a concentration of from 10-100 mM, Bst polymerase at an amount of from 1 U-12 U and 0.01%-1% 1.2 propanediol.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**FIG. 1** is a schematic of DNA probe of the invention.

**FIGS. 2A to 2F** shows amplification plots generated with the CT PB1 (FIG. 2A and FIG. 2D), GC glnA7 (FIG. 2B and FIG. 2E) and GC porA7 (FIG. 2C and FIG. 2F) primers in V6.21 buffer containing V13 (FIGS. 2A, 2B and 2C) or V6.21p buffer without V13 dye (FIGS. 2D, 2E and 2F).

**FIGS. 3A and 3B** are melt curve analyses of LAMP products generated with CT PB1 primers in the presence of CT PB1 internal probe conjugated with FAM. 100 pg per reaction of ATTC CT DNA standard was used as a positive control. A—normalized reporter plot, B—derivative reporter plot.

**FIGS. 4A and B** are melt curve analyses of LAMP product generated with GC glnA7 primers in the presence of GC glnA7 loop probe conjugated with JOE.

**FIGS. 5A and 5B** are melt curve analyses of LAMP product generated with GC porA7 primers in the presence of
GC porA7 loop probe conjugated with ALEXA546. 100 pg per reaction of ATTTC GC DNA standard was used as a positive control.

[0041] FIGS. 6A to 6D show the results of a test to confirm the DNA product specificity with a probe in loop mediated isothermal amplification.

[0042] FIG. 7 shows amplification plots generated with CT PB1 primers in V6.21 buffer containing V13 or V6.21p buffer without V13 dye but in the presence of CT PB1 terminal probe (complementary to loop region) with an internal C conjugated with FAM and 3’ terminat (3’dCC).

[0043] FIGS. 8A and 8B shows the amplification plots generated in V6.21p buffer containing ROX in the presence of CT PB1 primers and CT PB1 terminal probe with an internal cytosine conjugated with FAM (FIG. 8A), and universal primers and 3’UP probe with 3’ terminal cytosine conjugated with FAM (FIG. 8B).

[0044] FIGS. 9A to 9C show the amplification plots generated with CT PB1 primers in V6.21p buffer without V13 in the presence of CT PB1 internal probe with an internal C conjugated with FAM and a reference dye (ROX).

[0045] FIGS. 10A to 10C show the validation of CT PB1-FAM probe specificity. FIG. 10A shows amplification plots generated with CT PB1-FAM probe in the presence of CT DNA and CT primers.

[0046] FIGS. 11A and 11B shows the validation of CT PB1-FAM probe against APTIMA CT assay.

[0047] FIGS. 12A and 12B show the amplification plots generated in CT/NC multiplex with CT PB1-FAM+GC porA7-Alexa546 probes.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS OF THE INVENTION

Abbreviations

[0048] CT—Chlamydia trachomatis
GC—Neisseria gonorrhoeae
GlnA7—Glutamine synthetase
PorA7—porin protein A7
LAMP—loop mediated isothermal amplification
PCR—polymerase chain reaction.

[0049] The present invention will now be described, by way of example only, with reference to the following examples and figures.

LAMP Reaction

[0050] V13 based detection of the target CT and GT DNA by LAMP was performed using LAMP V6.21 reaction buffer developed by the Applicant. Probe based detection of the target DNA was performed in V6.21p (without V13). The LAMP primer concentrations were as follows: CT PB1-0.8 μM FIP & BIP primer, 0.2 μM F3 & B3 and 0.4 μM Loop primers, GC porA7 and GC glnA7—2 μM FIP & BIP primer, 0.25 μM F3 & B3 and 0.5 μM Loop primers. All probes were used at a final concentration of 0.625 μM. LAMP reactions were run for 60 mins at a constant temperature of 63°C using AB17500 real-time PCR machine. Readouts of the fluorescent signal were obtained in SybrGreen/FAM, Joe or Cy3 channel as appropriate.

Probe Sequences

[0051]

SEQ ID NO. 1: GTCGACGG[C-FAM]CCATAGAAT

SEQ ID NO. 2: TACGATAGAC[C-FAM]CCGGACGTTG (CT PB1-FAM internal)

SEQ ID NO. 3: TCGAACCA(C-FAM)[CGGTGAGA][ddC] (CT PB1-FAM terminal)

SEQ ID NO. 4: GCGAACRTA [C-ALEXA546] CAGCTATGATCC (GC porA7-joe loopF)

SEQ ID NO. 5: ATGTTCA [C-JOE] CATGCCTGAG (GC glnA7-ALEXA546 loopB)

or

SEQ ID NO. 6: CCA GGG TAT CTA ATC CTT TGG T G [C-FAM].

Target Sequences

[0052] The target DNA sequences used in the Examples are
-continued

2941 agcgagagt tggttcctta tctctagaaa tggagcagag tcaaatggtt gagcggagtg
3001 ttcgaatattt aacaggaata tctggtgaaa aattacaaag tgggatctc tctaaagaag
3061 aattatcgcg agttggagaa ggtagagaa agaaggttaca atcactatttt tatatacgtc
3121 ttgctacagc atataacgtt aaaaatggc aagagcccag tcctcttgctg aaaaaagag
3181 atcgatttagc ctgaaaatt attaatccct atgagttgctt atcacagttt catcagttttg
3241 atcgataaaa gcagatgata cttacaatct cagaactaag agttgtcttc tcaagagctaa
3301 aactctcat agttggttta tctcaccact tctgaaagtt tggagttagc gcaaatagag
3361 ttccagcgtc ttcagatttg cgagaagcg gcacaagagta gcagttgatttt
3421 tgttatcaaa tagagagaaa tctgcttctta atgtagatg aacggttggg aaaaatagac
3481 aggtcagcgt ttctccttcgg atttaccttt ctcctcctaa aatacgtaaa ttcaggccta
3541 tttaaagagt atagatcaat atagatacct tcaacttcct aaagagctca tcaaccctgag
3601 aatacaggaag tggagcaata cagctgtgca aacatttaaag atacacccca aatggctca
3661 aatagggttag gtagaagtt tttttctctta tttctcattc acggtcattl acggtcattl
3721 agctctcaca aagataagcg cagcaagagaa ttcacagggg aacaagaaag gggttgttttt
3781 atccagagg aaaaaatgtc ttcatttcgtt aaggtgattt ccagttcac gacaagttcctaa
3841 aacttgacctt ttagaggctt atggtgttta aagctgaaag catacttccg tgtaagttga
3901 gttttaguag aagaaggctg aagctgattt aagagccttg aaccattttt gacaacaacc
3961 tttaaagagt gtagaagtt tttttctctta tttctcattc acggtcattl acggtcattl
4021 ccaactcttt ttcctgacag taagttttta cggagtagct gaaagttttaa ctgacagaga
4081 aatataagtt atagatctaa cactctttta tttcaccatc aacaggaac ataaagatatt
4141 gtttaagttag ccagtcctta tcttggagaa tcaataaaggg tcaaccaggcc
4201 tgcacaatcgg tcaacagaaaa taaatttgctt tttcacaacct gctcagcagt atggtcaca
4261 atttacgcac agggtgattg cagcagtttg ccagagaga cgaataattt caagttcgatt
4321 tcttggccaa gaaaaccctt tataaaagtt taaagtttctt tatttttaag
4381 gatgaagcag catacattga caggaagctg gaaagaaacg cagttcagtt cgataaatgc
4441 tataaatgac gocatcctgg ttggtgtggtt atcagattag aacacagctt atattcgga
4501 ttcctcctaa ttcctcctaa aaaaaaatttct taaaatctt aaagagcgct ttaagaaat
4561 aacttactaa ttaaaagaag gaaatgca gtaatttattt aatattagc ccttctgtgaa
4621 cttaaaaaatt aatctaaat ctaaaaatttattg tttaaaaag taaaatttattg gaaatctttt
4681 gttttttttt gtaaatcctt gaaacctgtc tttgctgtga taatttcaaa gttggccaaag
4741 tgacagagcc gotcaagagac caacattatgc tcttggagaa aaatacaaca ccatgtgccag
4801 caaaccagc aagctgtggat ggaatatttt ttaaccattc caataatcctt ctaaacatgtg
4861 ctcctttttac aatgtgtggtt ttagggaaag aagttcactta aattttgtaa gaaagittg
4921 gaaatctaaat cccctgtgta attgtgtgtaa cttgtgtga tagttcagtt caagatatgtt
4981 tagttctata ccaaccagc cttcttctttag ctggtggttta aagttttttaa aacacccaaac
5041 ttcatattttt aatcttcggc aacggggttatt tcctcctcagt actcaattgga accttttaag
5101 gaggaacag tcattgaaag ttcacagcag ccacaaagg tcttgacagc atggttattag
5161 tctcaccgata tattttccga tcaagaatgg aagcgtcgcgt ttggtcagct ttggtagcag
5221 aagctgatgcc ttgcccctgtg gcagattttg atggtctactc atcagcccttt cctatttat
-continued

5281 gtagctcaag aaccaagatt actaatagc gattgactcc aaccaagtat tcattaagtg
5341 tagggcggttt aggaaagagtt gtagtatggg ttaaragcct ttctataaggg aatgtatatat
5401 taggaacta aactactctt aatgtatcttt ttttgaaagt aataacctcaa aaccaagtct
5461 aaccaatattt tataggattt tttttagattg tttttatattt agagaaaaaca gttgaatatta
5521 cgggggttttg tagcgaatat aaaaagaaaag tgaagggaagca tttttttatatgaaatag
5581 atgtgaaaaga gattttcccc gatttaggcc aaaaatagca agttaaaacag aaaaaaagctaa
5641 ctgctcttaaa cctctccctta gaaacctacc ataaaaagttg ctaactaatt ctaggaatgcc
5701 tctccaacaa aagaaatctt ttagggatttc tctgtctttt gaaaaaatagctc
5761 aaggtacacagt tttaaagcata aaaaaggtct tggattagct tattttgctct aagaaaagcaac
5821 tggbbbbbb caaatgatatag aacccatatct caaacgtttg agggtatgttg ccaacccattag
5881 tttttttttttttttt ggaattggga aacccaaactt ttcttttttaaagcggatgca
5941 actgggcocca attttttaggg aaaaaaggttt tcactgtga cctagacccg caatccaaatt
6001 tgtctttcttg aagggggttttttggcagtaa aataaccaaaaaa aaggggtccag gacatatgatgt
6061 aaccaacttaag ctaatcatttt gggagaaaaaa aaagagatgt gcggacctaa
6121 tcccttgatac attttcttacg aaccaagtta gagaatttcc gatcatatag aaccaagtta
6181 aaccaattatattttctttaagttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
SEQ ID No. 8: Neisseria gonorrhoeae partial porA gene for class 1 outer membrane protein, isolate G3 (GenBank: HG61886.1)
1 gcgcgcgcgc ggcgcgcgc gcccgcgcgc ccagagccag aggatctct ttcggtgt ggcgcgcgc
61 ttcggtgt ggcgcgcgc gcccgcgcgc ccagagccag aggatctct ttcggtgt ggcgcgcgc
121 gatcctgctg agcagacccac gttgctgtgcc gatcctgctg agcagacccac gttgctgtgcc gatcctgctg
181 gatcctgctg agcagacccac gttgctgtgcc gatcctgctg agcagacccac gttgctgtgcc gatcctgctg
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continued
The primer sequences used in the LAMP reaction are as follows:

CT Plasmid

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CTCTACAGTATCCCTTAGGC
F3

SEQ ID No. 11
TGAGCTTGGTCTCTTCTG
B3

SEQ ID No. 12
GCAGCTTGTAGTCCTCGTAGCTGCTG
FIP

SEQ ID No. 13
TGAAGCAGCGTGGAGCCCTTCTTCTATTGCAGTTG
BIP

SEQ ID No. 14
CGCGGGATGCGCTAAC
LP1

SEQ ID No. 15
TACAACGCTAGGCTG
LB1

GC porA7

SEQ ID No. 16
ACGAAAAACATGACCAGAG
F3

SEQ ID No. 17
AAGGCGGTTGAGAAAATCG
B3

SEQ ID No. 18
GATGGGCATGATGCAAGCCTGTCATGCTTC
FIP

SEQ ID No. 19
TGAATTGTGGCGAGCCGATCGAAATGCGAGACCGCGGAG
BIP

SEQ ID No. 20
CGGACATACATTACGCTATGCAA
LoopF1

GC glnA7

SEQ ID No. 21
TCTACTCTGGTGGTTGCTG
F3

SEQ ID No. 22
CTGACATGGAACTGCAA
B3

SEQ ID No. 23
CGAAGTCCACCACACCAGAATTTGGACCAAATGCGAG
FIP

SEQ ID No. 24
CTTCGAGGCAATATCTCCGATTGAGCTGCGTACGAT
BIP

SEQ ID No. 25
CAATGGAATCCGAGCC
LF

SEQ ID No. 26
ATTTACCAACATGGCGAG
LB

Buffer

The Applicant has developed a buffer system for use with the probes of the invention and is designated V6.21 (or V6.21p without V13 dye present) in the following Examples. The concentrations of the buffer components are after buffer reconstitution:

V6.21

4-10 mM dNTP's, 10 mM salt, 30 mM Tris pH8.8, 30 mM Trehalose, 1-8 U Bst polymerase, Dye and 0.05% propanediol.

PCR

CT/GC detection in clinical samples by real-time PCR was performed using APTIMA CT/GC multiplex (Gen-Probe) according to the manufacturer's instructions.

Agarose Gel Electrophoresis

DNA electrophoresis was conducted in 1% agarose gel 1×TAE buffer at 100V. LAMP DNA products were visualized with GelRed (Invitrogen) with transilluminator.

Example 1

FIGS. 2A to 2F shows amplification plots generated with the CT PB1 (FIG. 2A and FIG. 2D), GC glnA7 (FIG. 2B and FIG. 2E) and GC porA7 (FIG. 2C and FIG. 2F) primers in V6.21 buffer containing V13 (FIGS. 2A, 2B and 2C) or V6.21p buffer without V13 dye (FIGS. 2D, 2E and 2F). The target sequences shown in SEQ ID Nos. 7 to 9 with CT PB1 internal probe conjugated with FAM, GC glnA7 loop probe conjugated with JOE and GC porA7 loop probe conjugated with Alexa546 respectively. All reactions were performed for 60 mins at a constant temperature of 63 C with ABI7500 machine.

Example 2

FIGS. 3A and 3B are melt curve analyses of LAMP products generated with CT PB1 primers in the presence of CT PB1 internal probe conjugated with FAM. 100 pg per reaction of ATTC CT DNA standard was used as a positive control. A—normalized reporter plot, B—derivative reporter plot. Melt curve plots were generated based on the readings in FAM channel with ABI7500 machine.

Example 3

FIGS. 4A and B are melt curve analyses of LAMP product generated with GC glnA7 primers in the presence of GC glnA7 loop probe conjugated with JOE. 100 pg per reaction of ATTC GC DNA standard was used as a positive control. FIG. 4A shows a normalized reporter plot and FIG. 4B shows a derivative reporter plot. Melt curve plots were generated based on the readings in JOE channel with ABI7500 machine.
Example 4

[0065] FIGS. 5A and 5B are melt curve analyses of LAMP product generated with GC porA7 primers in the presence of GC porA7 loop probe conjugated with ALEXA546. 100 pg per reaction of ATTTC GC DNA standard was used as a positive control. FIG. 5A shows a normalized reporter plot, Fig. 4B shows a derivative reporter plot. Melt curve plots were generated based on the readouts in Cy3 channel with ABI17500 machine.

Example 5

[0066] FIGS. 6A to 6D) show the results of a test to confirm the DNA product specificity with a probe of the invention in loop mediated isothermal amplification. The late amplification time of the false positives (more than 30 mins after the lowest target DNA concentration detectable in the LAMP reaction (100 fg GC DNA) indicates that the unspecific amplification may be a result of primer dimer formation. The standard melt curve analysis does not allow to distinguish between the specific and unspecific product in this LAMP reaction, but the unspecific product may be recognized with the probe of the invention. GC DNA was amplified with GC porA7 primers and visualized with V13 dye or GC porA7-ALEXA546 probe as appropriate.

Example 6

[0067] FIG. 7 shows the amplification plots generated with CT PB1 primers in V6.21 buffer containing V13 or V6.21p buffer without V13 dye but in the presence of CT PB1 terminal probe (complementary to loop region) with an internal C conjugated with FAM and 3’ terminator (3’ddC). Despite a successful amplification of the target DNA confirmed by excitation of the V13 dye in the control reaction, CT PB1 probe with 3’ terminator did not generate a positive signal.

Example 7

[0068] FIGS. 8A and 8B shows the amplification plots generated in V6.21p buffer containing ROX in the presence of CT PB1 primers and CT PB1 terminal probe with an internal cytosine conjugated with FAM (FIG. 8A), and universal primers and 3’UP probe with 3’ terminal cytosine conjugated with FAM (FIG. 8B). The first line represents signals generated by ROX, and the second line corresponds to the signal generated in the FAM channel. Binding of the probe with an internally labeled C to the target DNA results in FAM excitation. Binding of the probe with a 3’ end C labeled to the target does not alter the FAM excitation state.

Example 8

[0069] FIGS. 9A to 9C show the amplification plots generated with CT PB1 primers in V6.21p buffer without V13 in the presence of CT PB1 internal probe with an internal C conjugated with FAM and a reference dye (ROX). Fig. 9A show raw data, readouts from the FAM channel in the first line and from the ROX channel in a second line. FIG. 9B shows amplification plots (generated in FAM channel) normalized to ROX. FIG. 9C shows derivative reporter melt curve plots.

Example 9

[0070] FIGS. 10A to 10C show the validation of CT PB1-FAM probe specificity. FIG. 10A shows amplification plots generated with CT PB1-FAM probe in the presence of CT DNA and CT primers. As a control, two sets of reactions were performed where unspecific genes, GC glnA7 and GC porA7 were amplified with the corresponding LAMP primers in the presence of CT PB1-FAM probe. In V6.21p buffer the amplification plots in the presence of CT PB1 probe in the FAM channel were generated only when CT DNA was present in the reaction and no signal was generated when unspecific genes (GC glnA7 and GC porA7) were amplified. No signal was also generated when an unspecific probe was used in a reaction where CT DNA was amplified with CT primers. FIG. 10C shows data obtained in an analogous experiment but conducted in V6.21 buffer containing an intercalating dye V31. FIG. 10C shows DNA products generated in the experiment described in Fig. 10A.

Example 10

[0071] FIGS. 11A and 11B shows the validation of CT PB1-FAM probe against APTIMA CT assay. Fifty clinical samples confirmed to be positive (n=29) (FIG. 11A) or negative (n=21) (FIG. 11B) for CT were tested in V6.21p buffer with CT PB1-FAM probe. Out of 50 samples 24 tested negative (FIG. 11A) and 26 tested positive (FIG. 11B) for CT with CT PB1-FAM probe. There was 86% agreement between the Aptima and CT PB-FAM tests.

Example 11

[0072] FIGS. 12A and 12B) shows the amplification plots generated in CT/GC multiplex with CT PB1-FAM+GC porA7-Alexa546 probes. CT and GC DNA was amplified in separate reactions or in conjugation in V6.21p buffer in the presence of CT PB1-FAM and GC porA7-Alexa546 probes. The readouts were taken in Cy3 (FIG. 12A) and FAM (FIG. 12B) channels. The experiment revealed that two DNA targets may be amplified and detected in a simultaneous reaction with FAM and Alexa546 labeled probes and that there was no cross reactivity between CT PB1 and GC porA7 primers and probes.

Example 12

[0073] Table 1 shows a comparison between V13 LAMP for CT and GC, CT/GC Aptima and CT/GC multiplex (CT PB1-FAM+GC porA7-Alexa546). DNA extracted from 136 clinical samples was tested with CT/GC Aptima multiplex, CT PB1 and GC porA7 primers in V6.21 buffer containing V13 or in a multiplex reaction in v6.21p buffer in the presence of CT PB1 and GC porA7 primers and CT PB1-FAM and GC porA7-Alexa546 probes. In a control experiment the samples were also tested in a simplex reaction with GC glnA7-joe probe. The table shows the agreement scores between the tests.
TABLE 1

Comparison between V13-based LAMP for CT and GC, CT/GC Aptima multiplex and CT/GC MAST multiplex (CT PB1-FAM + GC porA7-Alexa546). (Test on 136 clinical samples)

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<tr>
<th>Tests compared</th>
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<td>CT LAMP vs CT PB1-FAM in multiplex</td>
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<td>GC LAMP vs. GC porA7-Alexa546 in multiplex</td>
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<td>CT in multiplex vs CT Aptima</td>
<td>83%</td>
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<td>GC in multiplex vs GC Aptima</td>
<td>86%</td>
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CGACGACGACGACGACGACGACG
1. A probe for isothermal nucleic acid amplification comprising an oligonucleotide probe sequence complementary to a region of a target nucleic acid sequence, wherein said oligonucleotide probe sequence has only one fluorophore label and which label is bound to an internal cytosine base and wherein said oligonucleotide probe sequence does not have a 3' end terminator.

2. The probe of claim 1, wherein the cytosine base is substantially centrally disposed along the oligonucleotide's length except for positions 1-3 at the 3' end and position 1 at the 5' end.

3. The probe of claim 1, wherein the oligonucleotide probe sequence is a DNA sequence and the target nucleic acid sequence is a DNA sequence.

4. The probe of claim 1, wherein the fluorophore comprises any one or more selected from the group consisting of: FAM, JOE, TET, HEX, TAMRA, ROX, ALEXA and ATTO.

5. The probe of claim 1, comprising the following sequence:

5' Xn C*Xm 3'

wherein n is >1, m>3, X is nucleotide base; and * is fluorophore.

6. The probe of claim 5, wherein the nucleotide base is selected from the group consisting of A, T, C and G, n is more than 1 to 20 or less and m is more than 3 to 20 or less.

7. The probe of claim 1, comprising one or more of the following sequences:

SEQ ID NO: 2: TAAGATAAC[C-FAM]CCGACGTTG (CT PB1-FAM internal).

SEQ ID NO: 4: GCAGAACATA [C-ALEXA546] CAGCGATC (GC porA7-joe loopF), or

8. The probe of claim 1, wherein the target nucleic acid is from a micro-organism, fungi, yeast or virus.

9. The probe of claim 1, wherein the probe is configured to be used in loop-mediated isothermal nucleic acid amplification.

10. A method of detecting a target nucleic acid sequence in a sample, the method comprising:

amplifying a target nucleic acid in the sample to provide an amplified nucleic acid;
probing the amplified nucleic acid with a probe as claimed in claim 1; and

detecting the presence of the target nucleic acid, wherein an increases in fluorescence of the probe indicates the presence of the target nucleic acid in the sample.

11. The method of claim 10, wherein the target nucleic acid is from a micro-organism, fungi, yeast or virus.

12. The method of claim 10, wherein the target nucleic acid is from Chlamydia trachomatis or Neisseria gonorrhoeae.

13. A method of diagnosing Chlamydia and/or Gonorrhoea infection in a patient, the method comprising:

providing a sample derived from the patient;

adding one or more probes of claim 1 to the sample; and

detecting the presence of a nucleic acid derived from Chlamydia trachomatis and/or Neisseria gonorrhoeae, wherein an increase in the fluorescence of the probe indicates the presence of a Chlamydia trachomatis and/or Neisseria gonorrhoeae infection.

14. The method of claim 13, wherein a single type of probe specific for a nucleic acid from either Chlamydia trachomatis or Neisseria gonorrhoeae is added to the sample.

15. The method of claim 13, wherein at least two different probes are added to the sample wherein a first probe is labelled with a first fluorescent label and is specific for probing Chlamydia trachomatis nucleic acid and a second probe is labelled with a different fluorescent label to the first probe and is specific for probing Neisseria gonorrhoeae nucleic acid.

16. The method of claim 10, wherein the probes are provided in a buffer system comprising dNTPs at a concentration of from 1-10 mM, one or more salts at a concentration of each salt of from 2-20 mM, Tris pH 8.8 at a concentration of from 10-100 mM, Trehalose at a concentration of from 10-100 mM, BST polymerase at an amount of from 1 U-12 U and 0.01%-1% 1,2 propanediol.

17. The method of claim 16, wherein the one or more salts are selected from the group consisting of KCl, (NH₄)₂SO₄ and MgSO₄.

18. A kit for detecting a target nucleic acid comprising a probe as claimed in claim 1, a loop-mediated isothermal amplification reagent a buffer, an enzyme, dNTPs and one or more loop-mediated isothermal amplification primers.

19. The kit of claim 18, further comprising a positive and negative control.

20. The kit of claim 18, wherein the reagent buffer comprises dNTPs at a concentration of from 1-10 mM, one or more salts at a concentration of from 2-20 mM, Tris pH 8.8 at a concentration of from 10-100 mM, Trehalose at a concentration of from 10-100 mM, BST polymerase at an amount of from 1 U-12 U and 0.01%-1% 1,2 propanediol.

21. The kit of claim 20, wherein the one or more salts are selected from the group consisting of KCl, (NH₄)₂SO₄ and MgSO₄.

22. The probe of claim 4, wherein the fluorophore is FAM, Joe or Alexa546.