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(54) Title: POLYNUCLEOTIDES FOR THE AMPLIFICATION AND DETECTION OF NEISSERIA GONORRHOEAE

(57) Abstract: Disclosed herein are primers and probes related to the detection of *Neisseriagonorrhoeae* via nucleic acid amplification testing (NAAT), for example to amplify and determine the presence of *N.gonorrhoeae* nucleic acids present in test samples. Specifically the present disclosure describes primers and probes that bind to Cytochrome C or *ccpA* gene of *N.gonorrhoeae* for detection via loop mediated isothermal amplification (LAMP) and molecular beacon hybridization.

POLYNUCLEOTIDES FOR THE AMPLIFICATION AND DETECTION OF NEISSERIA GONORRHOEAE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. Patent Application No. 16/719,744, filed December 18, 2019, which is a continuation of U.S. Patent Application No. 16/523,609, filed July 26, 2019, which claims the benefit of U.S. Provisional Application No. 62/878,639, filed July 25, 2019, the contents of which are each incorporated by reference in their entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on July 23, 2020, is named TSM-052WO_SL.txt and is 17,533 bytes in size.

FIELD OF THE INVENTION

[0003] The present invention relates to the fields of molecular biology and nucleic acid chemistry. The invention provides methods and reagents for detecting pathogens, such as *Neisseria gonorrhoeae* and accordingly, also relates to the fields of medical diagnostics and prognostics. In particular, the invention relates to polynucleotides and methods for amplifying and detecting *Neisseria gonorrhoeae.*

BACKGROUND OF THE INVENTION

[0004] *Neisseria gonorrhoeae,* the etiological agent of gonorrhea, infects the urogenital tract with clinical signs of gonorrhea often overlapping with those of other sexually transmitted diseases (STDs). Infection, often asymptomatic in women, if left untreated can lead to more serious and permanent health related complications such as pelvic inflammatory disease (PID), chronic pelvic pain, tubal infertility, and life-threatening ectopic pregnancy. In men, the majority of urethral infections cause urethritis, occasionally resulting in epididymitis which can lead to infertility if not treated. Though not as common, asymptomatic infection rates among men are also significant. Among neonates, conjunctivitis can result in blindness. Among all three groups, untreated *N. gonorrhoeae* can disseminate leading to acute dermatitis, tenosynovitis syndrome and sepsis associated with arthritis, meningitis, or endocarditis.

[0005] *N. gonorrhoeae* has a global impact estimate of 106 million new cases annually. Worldwide, *N. gonorrhoeae* is the second most prevalent bacterial STD as well as the second most common notifiable communicable disease in the United States. The WHO estimates incidence of *N. gonorrhoeae* infection has been steadily rising since 1995, with an increase of 11.7% from 2005 to 2008. Compounding the clinical and increased incidence concerns is the categorization of *N. gonorrhoeae* as an immediate public health threat related to its antibiotic resistance profile, with 30% of strains estimated to carry resistance to one or more treatment antibiotics.

[0006] One of the main public health strategies in prevention and reduction of infectious disease is reducing person-to-person spread through screening, prompt identification and effective treatment. Imperative to this strategy are specific and sensitive diagnostics.

[0007] The performance of nucleic acid amplification tests (NAATs) as measured by sensitivity, specificity, and ease of specimen transport exceeds that of any other testing diagnostic currently available for diagnosing gonococcal infections. The US Centers for Disease Control (CDC) specifically recommends use of NAATs by clinical and disease control laboratories to detect gonorrhea with a few limited exceptions. Recommendations for the Laboratory-Based Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* — 2014. MMWR 2014;63(No. RR-2). Related to the sensitivity and specificity, these assays have provided for the use of less invasive specimen collection, which better facilitates infectious disease screening. Optimal recommended specimen types for NAATs include first catch urine from men and vaginal swabs from women.

[0008] FDA-cleared NAATs included Abbott RealTime CT/NG (Abbott m2000 system platform), Aptima COMBO or individual CT or GC assays (Hologic Panther system platform), BD ProbeTec assays (ET CT/GC Amplified DNA assay and Q^x CT or GC Amplified DNA assays and BD Viper system platform), Cepheid Xpert CT/NG assay (GeneXpert IV point of care device), and Roche Diagnostics CT/NG tests (cobas 4800 system platform). The Abbott, Aptima, BD, and Roche assays all include automation for sample preparation, target amplification, and detection. While this is a benefit from a sample preparation and limited hands on time perspective, each system platform translates into a large investment in capital equipment and requires at least 3 hours to reach a diagnostic result. The Cepheid assay and its accompanying device is the only point of care instrument, with reduced cost, spatial fingerprint, but still requires approximately 90 minutes to generate a diagnostic result.

[0009] What is needed, therefore, are new assays compatible with point of care devices that offer high sensitivity, significantly reduced time to answer, reduced equipment cost, and the potential for sample in answer out utilization.

SUMMARY

[0010] In some embodiments, provided herein is a composition comprising a set of polynucleotides selected from the group consisting of Set-1 through Set-27. In some embodiments, the composition further comprises a probe. In some embodiments, the probe comprises a label. In some embodiments, the probe is a labeled polynucleotide.

[0011] In some embodiments, the probe is a labeled polynucleotide having a sequence selected from the group consisting of SEQ ID NO: 73-76 and the set of polynucleotides is selected from Sets 12-15.

[0012] In some embodiments, the label is a fluorophore. In some embodiments, the fluorophore is covalently attached to a terminus of the polynucleotide. In some embodiments, the probe is a molecular beacon comprising a quencher. In some embodiments, the fluorophore is FAM and the quencher is BHQ1. In other embodiments, the fluorophore is ATTO 565 or Alexa 594 and the quencher is BHQ1 or BHQ2.

[0013] Also provided herein is a molecular beacon comprising a fluorophore, a quencher and a polynucleotide, wherein the polynucleotide is selected from the group consisting of SEQ ID NOs: 73-76 and the set of polynucleotides is selected from Sets 12-15. In some embodiments, the fluorophore is FAM and the quencher is BHQ1. In other embodiments, the fluorophore is ATTO 565 or Alexa 594 and the quencher is BHQ1 or BHQ2.

[0014] Also provided herein is a method of detecting *Neisseria gonorrhoeae* in a test sample, the method comprising: (a) extracting nucleic acid from the test sample; (b) amplifying a target sequence by reacting the nucleic acid extracted in step (a) with a reaction mixture comprising a strand displacement DNA polymerase and a sequence-specific primer set, wherein said sequence-specific primer set is selected from the group consisting of Set-1 through Set-27; and (c) detecting the presence or absence of an amplified product of step (b); wherein the presence of said amplification product is indicative of the presence of *Neisseria gonorrhoeae* in the test sample.

[0015] In some embodiments of the method of detecting *Neisseria gonorrhoeae* in a test sample, the amplification in step (b) of the target sequence is performed at between about 60° C and 67° C for less than 30 minutes. In some embodiments, the amplification step is performed for less than 15 minutes. In some embodiments, the amplification step is performed for less than ten minutes.

[0016] In some embodiments of the method of detecting *Neisseria gonorrhoeae* in a test sample, detecting the presence or absence of the amplification product comprises hybridizing the amplified product with a probe comprising a polynucleotide attached to a label.

[0017] In some embodiments of the method of detecting *Neisseria gonorrhoeae* in a test sample, the polynucleotide comprises a sequence selected from the group consisting of SEQ ID NO: 73-76 and the sequence-specific primer set is selected from Sets 12-15.

[0018] In some embodiments of the method of detecting *Neisseria gonorrhoeae* in a test sample, the probe is a molecular beacon. In some embodiments, the reaction mixture further comprises a reverse transcriptase. In some embodiments, *Neisseria gonorrhoeae* is present in the test sample at a concentration of $\leq$ 100 CFU/mL. In some embodiments, *Neisseria gonorrhoeae* is present in the test sample at a concentration of $\leq$ 10 CFU/mL.

[0019] Also provided herein, according to some embodiments of the invention, is a kit comprising a composition comprising a set of polynucleotides selected from the group consisting of Set-1 through Set-27 and amplification reagents. In some embodiments, the amplification reagents comprise a strand displacement polymerase. In some embodiments, the kit further comprises a probe.

[0020] Also provided herein is a method of detecting *Neisseria gonorrhoeae* in a test sample, the method comprising: (a) extracting nucleic acid from the test sample; (b) amplifying a target sequence by reacting the nucleic acid extracted in step (a) for less than twenty minutes with a reaction mixture comprising a strand displacement DNA polymerase and a sequence-specific LAMP primer set; and (c) detecting the presence or absence of an amplified product of step (b); wherein the presence of said amplification product is indicative of the presence of *Neisseria gonorrhoeae* in the test sample.

[0021] In some embodiments of the method, the nucleic acid is reacted with the reaction mixture for less than fifteen minutes. In some embodiments of the method, the target sequence is located in the cytochrome C peroxidase (*ccpA*) gene of *Neisseria gonorrhoeae*.

[0022] In some embodiments of the method, *Neisseria gonorrhoeae* is present in the test sample at a concentration of ≤ 100 CFU/mL. In some embodiments of the method, *Neisseria gonorrhoeae* is present in the test sample at a concentration of ≤ 10 CFU/mL.

[0023] In some embodiments of the method, the test sample comprises one or more other microorganisms in addition to *Neisseria gonorrhoeae*, and wherein the target sequence from *Neisseria gonorrhoeae* is preferentially amplified over a polynucleotide sequence from the one or more other microorganisms.

[0024] In some embodiments, the invention provides a nucleic acid sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8% or at least 99.9% identical to SEQ ID NOs 1-76 and methods of using those nucleic acid sequences to detect *Neisseria gonorrhoeae* in a test sample.

DETAILED DESCRIPTION

[0025] Detecting low concentrations of species (down to a few molecules or microorganisms in a sample) is a challenge in medicine. The present invention relates to the selective detection of *Neisseria gonorrhoeae*. In particular, based on new detection strategies utilizing nucleic acid amplification, particularly RT-LAMP, and molecular beacon detection, *N. gonorrhoeae* infections can be diagnosed using the methods and reagents described herein. Using RNA (either ribosomal RNA (rRNA) or messenger RNA) as the target regions provides multiple copies of the target per *N. gonorrhoeae* genome. Accordingly, this facilitates the detection of *N. gonorrhoeae* in samples utilizing the approaches described herein relative to techniques that target genomic DNA, even when present in multiple copies per genome. In addition, the molecular beacon detection reagents described herein provide additional specificity, failing to bind, in most cases, to off target amplified DNA, thereby minimizing the occurrence of, e.g., false positives. This specificity is illustrated in, *inter alia*, Example 3 (Tables 4 and 5) provided below. Many other features of the invention are also described herein.

[0026] As used herein, “nucleic acid” includes both DNA and RNA, including DNA and RNA containing non-standard nucleotides. A “nucleic acid” contains at least one polynucleotide (a “nucleic acid strand”). A “nucleic acid” may be single-stranded or double-stranded. The term “nucleic acid” refers to nucleotides and nucleosides which make up, for example, deoxyribonucleic acid (DNA) macromolecules and ribonucleic acid (RNA) macromolecules. The most common nucleic acids are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). It should be further understood that the present invention can be used for biological sequences containing artificial nucleotides such as peptide nucleic acid (PNA), morpholino, locked nucleic acid (LNA), glycol nucleic acid (GNA) and threose nucleic acid (TNA), among others. Preferably, the artificial nucleotides are locked nucleic acid molecules, including [alpha]-L-LNAs. LNAs comprise ribonucleic acid analogues wherein the ribose ring is "locked" by a methylene bridge between the 2'-oxygen and the 4'-carbon-i.e., oligonucleotides, containing at least one LNA monomer, that is, one 2'-O,4'-C-methylene-β-D-ribofuranosyl nucleotide. LNA bases form standard Watson-Crick base pairs but the locked configuration increases the rate and stability of the base-pairing reaction (Jepsen et al., Oligonucleotides, 14, 130-146 (2004)).

[0027] As used herein, a "polynucleotide" refers to a polymeric chain containing two or more nucleotides, which contain deoxyribonucleotides, ribonucleotides, and/or their analog, such as those containing modified backbones (e.g. peptide nucleic acids (PNAs) or phosphorothioates) or modified bases. "Polynucleotides" includes primers, oligonucleotides, nucleic acid strands, etc. A polynucleotide may contain standard or non-standard nucleotides. Thus, the term includes mRNA, tRNA, rRNA, ribozymes, DNA, cDNA, recombinant nucleic acids, branched nucleic acids, plasmids, vectors, probes, primers, etc. Typically, a polynucleotide contains a 5' phosphate at one terminus ("5' terminus") and a 3' hydroxyl group at the other terminus ("3' terminus") of the chain. The most 5' nucleotide of a polynucleotide may be referred to herein as the "5' terminal nucleotide" of the polynucleotide. The most 3' nucleotide of a polynucleotide may be referred to herein as the "3' terminal nucleotide" of the polynucleotide. Where nucleic acid of the invention takes the form of RNA, it may or may not have a 5' cap.

[0028] LAMP is a nucleic acid amplification method that relies on auto-cycle strand-displacement DNA synthesis performed by a Bst DNA polymerase or another strand displacement polymerase. The amplified products are stem-loop structures with several repeated sequences of the target and multiple loops. The principal merit of this method is that denaturation of the DNA template is not required, and thus the LAMP reaction can be conducted under isothermal conditions (ranging from 60 to 67° C). LAMP requires only one enzyme and four types of primers that recognize six distinct hybridization sites in the target sequence. The reaction can be accelerated by the addition of two additional primers. The method produces a large amount of amplified product, resulting in easier detection, such as detection by visual judgment of the turbidity or fluorescence of the reaction mixture.

[0029] In brief, the reaction is initiated by annealing and extension of a pair of ‘loop-forming’ primers (forward and backward inner primers, FIP and BIP, respectively), followed by annealing and extension of a pair of flanking primers (F3 and B3). Extension of these primers results in strand-displacement of the loop-forming elements, which fold up to form terminal hairpin-loop structures. Once these key structures have appeared, the amplification process becomes self-sustaining, and proceeds at constant temperature in a continuous and exponential manner (rather than a cyclic manner, like PCR) until all of the nucleotides (dATP, dTTP, dCTP & dGTP) in the reaction mixture have been incorporated into the amplified DNA, or the chemical reaction is otherwise exhausted. Optionally, an additional pair of primers can be included to accelerate the reaction. These primers, termed “loop primers,” hybridize to non-inner primer bound terminal loops of the inner primer dumbbell-shaped products.

[0030] The term “primer” as used herein refers to an oligonucleotide, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of primer extension product which is complementary to a nucleic acid strand (template) is induced, i.e., in the presence of nucleotides and an agent for polymerization, such as DNA polymerase, and at a suitable temperature and pH.

[0031] LAMP allows amplification of target DNA sequences with higher sensitivity and specificity than PCR, often with reaction times below 30 minutes, which is equivalent to the fastest real-time PCR tests. The target sequence which is amplified is typically 200-300 base-pairs (bp) in length, and the reaction relies upon recognition of between 120 bp and 180 bp of this sequence by several primers simultaneously during the amplification process. This high level of stringency makes the amplification highly specific, such that the appearance of amplified DNA in a reaction occurs only if the entire target sequence was initially present.

[0032] Applications for LAMP have been further extended to include detection of RNA molecules by addition of Reverse Transcriptase enzyme (RT). By including RNA detection, the types of targets for which LAMP can be applied are also expanded and add the ability to additionally target RNA-based viruses, important regulatory non-coding RNA (sRNA, miRNA), and RNA molecules that have been associated with particular disease or physiological states. The ability to detect RNA also has the potential to increase assay sensitivity, for instance in choosing highly expressed, stable, and/or abundant messenger

RNA (mRNA) or ribosomal RNA (rRNA) targets. This preliminary phase of amplification involves the reverse transcription of RNA molecules to complementary DNA (cDNA). The cDNA then serves as template for the strand displacing DNA polymerase. Use of a thermostable RT enzyme (i.e., NEB RTx) enables the reaction to be completed at a single temperature and in a one step, single mix reaction.

[0033] A "target sequence," as used herein, means a nucleic acid sequence of *Neisseria gonorrhoeae*, or complement thereof, that is amplified, detected, or both amplified and detected using one or more of the polynucleotides herein provided. Additionally, while the term target sequence sometimes refers to a double stranded nucleic acid sequence, those skilled in the art will recognize that the target sequence also can be single stranded, *e.g.*, RNA. A target sequence may be selected that is more or less specific for a particular organism. For example, the target sequence may be specific to an entire genus, to more than one genus, to a species or subspecies, serogroup, auxotype, serotype, strain, isolate or other subset of organisms.

[0034] The speed, specificity and sensitivity of the primers/probe compositions and method described herein result from several aspects. Exemplary primers for use in the compositions and methods according to the present invention include:

Table 1 - LAMP primers

Seq ID	Sequence (5′ to 3′)
SEQ ID NO: 1	CATAAGGAATACACGATGTCTTTC
SEQ ID NO: 2	GATTTTCTGCATTTCTTCGACA
SEQ ID NO: 3	AAGCAGGAGAAGCATCGCCTATTGGCATTGTCTTCACTGT
SEQ ID NO: 4	CGCCCGAAGACCAAGACCGTCGGCAAAGGTTGGAATA
SEQ ID NO: 5	GCCGCAGACTTTTCCTGA
SEQ ID NO: 6	TTTGAAACGCGCGCAAG
SEQ ID NO: 7	AAGGCAACGTCAACGC
SEQ ID NO: 8	GTCAGCACGGCCTTTG
SEQ ID NO: 9	CACCGTTGTGGCAGGCACTGAGCGAACAGGAACG
SEQ ID NO: 10	CAACCTTGGAGGCACGACCTGAATTTCCAATACGGCCC
SEQ ID NO: 11	GTTGTCCATGAACGCGC
SEQ ID NO: 12	CAGAAATTCGGTCTGGTCCA
SEQ ID NO: 13	TATTCCAACCTTTGCCGAC
SEQ ID NO: 14	GCCGAACTGCCCTTTG
SEQ ID NO: 15	CTTTGGAAAGGCGTGGTTCATACCCGTTTACCGAAGAACAGG
SEQ ID NO: 16	CAAAGGCAATACCGTAAGCTGCCATATTGTCCACACCGGC
SEQ ID NO: 17	CAGAGTTGGTGTCCGAGTT
SEQ ID NO: 18	CTTGCCACAACCTTGCTTC
SEQ ID NO: 19	TATTTCCACAACGGCAGC

Seq ID	Sequence (5' to 3')
SEQ ID NO: 20	GGCTTAGATTCCATCGGTG
SEQ ID NO: 21	CCACATCTTCTTTCGGAATGTCTTTTGGGAGCTGGATAAGGC
SEQ ID NO: 22	GTGGATAACATCGTCGTATTCCTGACAGTTCCGGCATCGTG
SEQ ID NO: 23	CAATTGCGCCTTACCCATG
SEQ ID NO: 24	CTTTCCGGCAATGTTTCCG
SEQ ID NO: 25	TTGTCGCGTTTCGGATC
SEQ ID NO: 26	CCAACCTTTGCCGACTG
SEQ ID NO: 27	CATCACTACCGCATTGGG
SEQ ID NO: 28	CAGACCGAATTTCTGGAAGG
SEQ ID NO: 29	GCTCAGGGCGTTGACGTGTTTGAGCGTACCCTGC
SEQ ID NO: 30	GCGCGTTCATGGACAACGCGTGCCTCCAAGGTTG
SEQ ID NO: 31	CCATTTGGTCGGCGTCA
SEQ ID NO: 32	GCTGTATTGCCTGCCACA
SEQ ID NO: 33	CTTAGATTCCATCGGTGCG
SEQ ID NO: 34	GGCAATGTTTCCGAATCAGC
SEQ ID NO: 35	GCGATGCTTCTCCTGC
SEQ ID NO: 36	GTGTCCGAGTTTGACCTG
SEQ ID NO: 37	GAAGCGGAAGGAACGGCTTTTCCGAGACCGAAGCG
SEQ ID NO: 38	CTCGCCCGAAGACCAAGACTCTGCATTTCTTCGACAGTC
SEQ ID NO: 39	GGCCTGTACTTGGGAAGC
SEQ ID NO: 40	GCGCAAGGTGTATTCCAAC
SEQ ID NO: 41	GTGTCCGAGTTTGACCTGT
SEQ ID NO: 42	CAACCTTGCTTCCGCC
SEQ ID NO: 43	TCATTCGCCATTTCCACC
SEQ ID NO: 44	ATGCGGTAGGCGAGTTGCGTGGACAATATGCCGACC
SEQ ID NO: 45	GGGCAGCCAGTTTTGGGAGATTCACCAAAGGCCCG
SEQ ID NO: 46	CCCTTTGTGCCCCTGAC
SEQ ID NO: 47	GTGCCGCCGATGTTGA
SEQ ID NO: 48	AACTCGGACACCAACTC
SEQ ID NO: 49	CATTCAATGCGGTAGGC
SEQ ID NO: 50	AGCAAGGTTGTGGCAAGAGGGTATGAACCACGCCTT
SEQ ID NO: 51	CGCCGGTGTGGACAATGAACTGCCCTTTGTGC
SEQ ID NO: 52	CAGCTTACGGTATTGCCTT
SEQ ID NO:53	ATGCCGACCAGTCAGG
SEQ ID NO: 54	ATATGCCGACCAGTCAG
SEQ ID NO: 55	GGGAACTTTGGCGATTT
SEQ ID NO: 56	AGCAGCGCAGCATTCAATGCACAAAGGGCAGTTC
SEQ ID NO: 57	TGTTGAAGAACAGGCTGGCGCGAATCATTCGCCATT
SEQ ID NO: 58	GTAGGCGAGTTGCGTC
SEQ ID NO: 59	TTGGTGAATCCGGTGGA
SEQ ID NO: 60	GAGTTGCGTCCGCCGAACTGCCTCTTGCCACAACCTTGCTTCCG
SEQ ID NO: 61	ACCGCATTGAATGCTGCGCTGCTGCCCGCCAGCCTGTTCTTC
SEQ ID NO: 62	TGACTGGTCGGCATATTGTCCACAC
SEQ ID NO: 63	CGGACGTGCCGCCGATGTT
SEQ ID NO: 64	CCTTTCCAAAGGCAATACCGTAAGC

Seq ID	Sequence (5′ to 3′)
SEQ ID NO: 65	TCGCCATTTCCACCGGATTCAC
SEQ ID NO: 66	TGCGGTAGGCGAGTTGCGGGACAATATGCCGACCAGT
SEQ ID NO: 67	TTGAATGCTGCGCTGCTGGCAGCCTGTTCTTCAACATCG
SEQ ID NO: 68	CGAACTGCCCTTTGTGCC
SEQ ID NO: 69	TTGGGACGGACGTGCC
SEQ ID NO: 70	TGCCACAACCTTGCTTCC
SEQ ID NO: 71	CGAATCATTCGCCATTTCCA
SEQ ID NO: 72	CTTTGGAAAGGCGTGGTTCATACAAATCCGTCCGTTTACCG

[0035] Detection of the LAMP amplified products can be achieved via a variety of methods. In a preferred embodiment, detection of product is conducted by adding a fluorescently-labeled probe to the primer mix. The term used herein “probe” refers to a single-stranded nucleic acid molecule comprising a portion or portions that are complementary, or substantially complementary, to a target sequence. In certain implementations, the fluorescently-labeled probe is a molecular beacon.

[0036] As used herein, “molecular beacon” refers to a single stranded hairpin-shaped oligonucleotide probe designed to report the presence of specific nucleic acids in a solution. A molecular beacon consists of four components; a stem, hairpin loop, end-labelled fluorophore and opposite end-labelled quencher (Tyagi et al., (1998) Nature Biotechnology 16:49-53). When the hairpin-like beacon is not bound to a target, the fluorophore and quencher lie close together and fluorescence is suppressed. In the presence of a complementary target nucleotide sequence, the stem of the beacon opens to hybridize to the target. This separates the fluorophore and quencher, allowing the fluorophore to fluoresce. Alternatively, molecular beacons also include fluorophores that emit in the proximity of an end-labelled donor. “Wavelength-shifting Molecular Beacons” incorporate an additional harvester fluorophore enabling the fluorophore to emit more strongly. Current reviews of molecular beacons include Wang et al., 2009, *Angew Chem Int Ed Engl*, 48(5):856-870; Cissell et al., 2009, *Anal Bioanal Chem* 393(1):125-35; Li et al., 2008, *Biochem Biophys Res Comm* 373(4):457-61; and Cady, 2009, *Methods Mol Biol* 554:367-79.

[0037] In one implementation, the molecular beacon comprises a fluorophore, a quencher, and a polynucleotide, wherein the polynucleotide comprises a sequence selected from the group consisting of SEQ ID NOS: 73-76. In some embodiments, the polynucleotide comprises a sequence selected from the group consisting of nucleotides 6-22 of SEQ ID NO: 73, nucleotides 5-24- of SEQ ID NO: 74, nucleotides 3-21 of SEQ ID NO: 75, and nucleotides 3-24 of SEQ ID NO: 76. The polynucleotides having the sequences described above can include one or more non-natural nucleosides or linkages, such as peptide nucleic acid (PNA), morpholino, locked nucleic acid (LNA), glycol nucleic acid (GNA) and threose nucleic acid (TNA), among others. In some embodiments, the polynucleotide of the molecular beacon comprises one to six locked nucleic acids. In a preferred embodiment, the polynucleotide of the molecular beacon comprises three locked nucleic acids. In another preferred embodiment, the polynucleotide of the molecular beacon comprises four locked nucleic acids.

[0038] The term “label” as used herein means a molecule or moiety having a property or characteristic which is capable of detection and, optionally, of quantitation. A label can be directly detectable, as with, for example (and without limitation), radioisotopes, fluorophores, chemiluminophores, enzymes, colloidal particles, fluorescent microparticles and the like; or a label may be indirectly detectable, as with, for example, specific binding members. It will be understood that directly detectable labels may require additional components such as, for example, substrates, triggering reagents, quenching moieties, light, and the like to enable detection and/or quantitation of the label. When indirectly detectable labels are used, they are typically used in combination with a “conjugate”. A conjugate is typically a specific binding member that has been attached or coupled to a directly detectable label. Coupling chemistries for synthesizing a conjugate are well known in the art and can include, for example, any chemical means and/or physical means that does not destroy the specific binding property of the specific binding member or the detectable property of the label. As used herein, “specific binding member” means a member of a binding pair, i.e., two different molecules where one of the molecules through, for example, chemical or physical means specifically binds to the other molecule. In addition to antigen and antibody specific binding pairs, other specific binding pairs include, but are not intended to be limited to, avidin and biotin; haptens and antibodies specific for haptens; complementary nucleotide sequences; enzyme cofactors or substrates and enzymes; and the like.

[0039] The molecular beacon can be composed of nucleic acid only such as DNA or RNA, or it can be composed of a peptide nucleic acid (PNA) conjugate. The fluorophore can be any fluorescent organic dye or a single quantum dot. The quenching moiety desirably quenches the luminescence of the fluorophore. Any suitable quenching moiety that quenches the luminescence of the fluorophore can be used. A fluorophore can be any fluorescent marker/dye known in the art. Examples of suitable fluorescent markers include, but are not limited to, Fam, Hex, Tet, Joe, Rox, Tamra, Max, Edans, Cy dyes such as Cy5, Fluorescein, Coumarin, Eosine, Rhodamine, Bodipy, Alexa, Cascade Blue, Yakima Yellow, Lucifer Yellow, Texas Red, and the family of ATTO dyes. A quencher can be any quencher known in the art. Examples of quenchers include, but are not limited to, Dabcyl, Dark Quencher, Eclipse Dark Quencher, ElleQuencher, Tamra, BHQ and QSY (all of them are Trade-Marks). The skilled person would know which combinations of dye/quencher are suitable when designing a probe. In an exemplary embodiment, fluorescein (FAM) is used in conjunction with Blackhole Quencher™ (BHQ™)(Novato, Calif.). Binding of the molecular beacon to amplified product can then be directly, visually assessed. Alternatively, the fluorescence level can be measured by spectroscopy in order to improve sensitivity.

[0040] A variety of commercial suppliers produce standard and custom molecular beacons, including Abingdon Health (UK; www.abingdonhealth.com), Attostar (US, MN; www.attostar.com), Biolegio (NLD; www.biolegio.com), Biomers.net (DEU; www.biomers.net), Biosearch Technologies (US, CA; www.biosearchtech.com), Eurogentec (BEL; www.eurogentec.com), Gene Link (US, NY; www.genelink.com) Integrated DNA Technologies (US, IA; www.idtdna.com), Isogen Life Science (NLD; www.isogen-lifescience.com), Midland Certified Reagent (US, TX; www.oligos.com), Eurofins (DEU; www.eurofinsgenomics.eu), Sigma-Aldrich (US, TX; www.sigmaaldrich.com), Thermo Scientific (US, MA; www.thermoscientific.com), TIB MOLBIOL (DEU; www.tib-molbiol.de), TriLink Bio Technologies (US, CA; www.trilinkbiotech.com). A variety of kits, which utilize molecular beacons are also commercially available, such as the Sentinel™ Molecular Beacon Allelic Discrimination Kits from Stratagene (La Jolla, Calif.) and various kits from Eurogentec SA (Belgium, eurogentec.com) and Isogen Bioscience BV (The Netherlands, isogen.com).

[0041] The oligonucleotide probes and primers of the invention are optionally prepared using essentially any technique known in the art. In certain embodiments, for example, the oligonucleotide probes and primers described herein are synthesized chemically using essentially any nucleic acid synthesis method, including, e.g., according to the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), Tetrahedron Setts. 22(20):1859-1862, which is incorporated by reference, or another synthesis technique known in the art, e.g., using an automated synthesizer, as described in Needham-VanDevanter et al. (1984) Nucleic Acids Res. 12:6159-6168, which is incorporated by reference. A wide variety of equipment is commercially available for automated oligonucleotide synthesis. Multi-nucleotide synthesis approaches (e.g., tri-nucleotide synthesis, etc.) are also optionally utilized. Moreover, the primer nucleic acids described herein optionally include various modifications. To further illustrate, primers are also optionally modified to improve the specificity of amplification reactions as described in, e.g., U.S. Pat. No. 6,001,611, issued Dec. 14, 1999, which is incorporated by reference. Primers and probes can also be synthesized with various other modifications as described herein or as otherwise known in the art.

[0042] In addition, essentially any nucleic acid (and virtually any labeled nucleic acid, whether standard or non-standard) can be custom or standard ordered from any of a variety of commercial sources, such as Integrated DNA Technologies, the Midland Certified Reagent Company, Eurofins, Biosearch Technologies, Sigma Aldrich and many others.

[0043] Test samples are generally derived or isolated from subjects, typically mammalian subjects, more typically human subjects, suspected of having a *N. gonorrhoeae* infection. Exemplary samples or specimens include blood, plasma, serum, urine, synovial fluid, seminal fluid, seminal plasma, prostatic fluid, vaginal fluid, cervical fluid, uterine fluid, cervical scrapings, amniotic fluid, anal scrapings, mucus, sputum, tissue, and the like. Essentially any technique for acquiring these samples is optionally utilized including, e.g., scraping, venipuncture, swabbing, biopsy, or other techniques known in the art.

[0044] The term “test sample” as used herein, means a sample taken from an organism or biological fluid that is suspected of containing or potentially contains a target sequence. The test sample can be taken from any biological source, such as for example, tissue, blood, saliva, sputa, mucus, sweat, urine, urethral swabs, cervical swabs, vaginal swabs, urogenital or anal swabs, conjunctival swabs, ocular lens fluid, cerebral spinal fluid, milk, ascites fluid, synovial fluid, peritoneal fluid, amniotic fluid, fermentation broths, cell cultures, chemical reaction mixtures and the like. The test sample can be used (i) directly as obtained from the source or (ii) following a pre-treatment to modify the character of the sample. Thus, the test sample can be pre-treated prior to use by, for example, preparing plasma or serum from blood, disrupting cells or viral particles, preparing liquids from solid materials, diluting viscous fluids, filtering liquids, distilling liquids, concentrating liquids, inactivating interfering components, adding reagents, purifying nucleic acids, and the like.

[0045] Advantageously, the invention enables reliable rapid detection of *Neisseria gonorrhoeae* in a clinical sample, such as a urine sample.

[0046] To further illustrate, prior to analyzing the target nucleic acids described herein, those nucleic acids may be purified or isolated from samples that typically include complex mixtures of different components. Cells in collected samples are typically lysed to release the cell contents. For example, *N. gonorrhoeae* and other cells in the particular sample can be lysed by contacting them with various enzymes, chemicals, and/or lysed by other approaches known in the art, which degrade, e.g., bacterial cell walls. In some embodiments, nucleic acids are analyzed directly in the cell lysate. In other embodiments, nucleic acids are further purified or extracted from cell lysates prior to detection. Essentially any nucleic acid extraction methods can be used to purify nucleic acids in the samples utilized in the methods of the present invention. Exemplary techniques that can be used to purifying nucleic acids include, e.g., affinity chromatography, hybridization to probes immobilized on solid supports, liquid-liquid extraction (e.g., phenol-chloroform extraction, etc.), precipitation (e.g., using ethanol, etc.), extraction with filter paper, extraction with micelle-forming reagents (e.g., cetyl-trimethyl-ammonium-bromide, etc.), binding to immobilized intercalating dyes (e.g., ethidium bromide, acridine, etc.), adsorption to silica gel or diatomic earths, adsorption to magnetic glass particles or organo-silane particles under chaotropic conditions, and/or the like. Sample processing is also described in, e.g., U.S. Pat. Nos. 5,155,018, 6,383,393, and 5,234,809, which are each incorporated by reference.

[0047] A test sample may optionally have been treated and/or purified according to any technique known by the skilled person, to improve the amplification efficiency and/or qualitative accuracy and/or quantitative accuracy. The sample may thus exclusively, or essentially, consist of nucleic acid(s), whether obtained by purification, isolation, or by chemical synthesis. Means are available to the skilled person, who would like to isolate or purify nucleic acids, such as DNA, from a test sample, for example to isolate or purify DNA from cervical scrapes (e.g., QIAamp-DNA Mini-Kit; Qiagen, Hilden, Germany).

EXAMPLES:

[0048] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1: Target Selection, Sequence Analysis and Assay Design

[0049] Sequences for *Neisseria gonorrhoeae* and closely related species including *Neisseria meningitidis*, *Neisseria lactamica*, *Neisseria sicca* and *Neisseria cinerea* were obtained from the National Center for Biotechnology Information (NCBI) or Pathosystems Resource Integration Center (PATRIC) databases. Sequences were aligned using Clustal Omega (Sievers, et al. (2011). Molecular Systems Biology 7:539) or MAFFT (Katoh, Standley 2013. *Molecular Biology and Evolution* 30:772-780) and regions unique to *N. gonorrhoeae* were selected for primer and molecular beacon probe design.

[0050] Primer/probe-based detection assays were designed to utilize isothermal loop mediated amplification targeting RNA through the addition of a Reverse transcriptase (RT-LAMP) to the reaction. A molecular beacon probe with 5' fluorophore/3' quencher modifications, 6-Carboxyfluorescein and Black Hole Quencher 1, was included to provide target-specific fluorescent detection. *N. gonorrhoeae* RT-LAMP primer sets (Table 1 and Table 2) were designed using a combination of software programs including PremierBiosoft’s LAMP Designer, Beacon Designer, an in-house script and manual designs. Resultant predicted amplicons were additionally Blasted against the NCBI nucleotide database, including the human transcriptome, and against individual non-*gonorrhoeae* species within the genus *Neisseria* to further predict assay specificity.

[0051] The inventive primer sets are summarized in Table 2, which include, at a minimum, a forward inner primer (FIP) and backward inner primer (BIP). Additionally, the primer sets typically also include at least two additional primers selected from the forward outer primer (F3), backward outer primer (B3), forward loop primer (LF) and backward loop primer (LB).

TABLE 2 - LAMP Primer Sets

Set	F3	B3	FIP	BIP	LF	LB
Set-1	SEQ ID NO: 1	SEQ ID NO: 2	SEQ ID NO:3	SEQ ID NO: 4	SEQ ID NO: 5	SEQ ID NO: 6
Set-2	SEQ ID NO: 7	SEQ ID NO: 8	SEQ ID NO: 9	SEQ ID NO: 10	SEQ ID NO: 11	SEQ ID NO: 12
Set-3	SEQ ID NO: 13	SEQ ID NO: 14	SEQ ID NO: 15	SEQ ID NO: 16	SEQ ID NO: 17	SEQ ID NO: 18
Set-4	SEQ ID NO: 19	SEQ ID NO: 20	SEQ ID NO: 21	SEQ ID NO: 22	SEQ ID NO: 23	SEQ ID NO: 24
Set-5	SEQ ID NO: 13	SEQ ID NO: 25	SEQ ID NO: 9	SEQ ID NO: 10	SEQ ID NO: 11	SEQ ID NO: 12
Set-6	SEQ ID NO: 26	SEQ ID NO: 14	SEQ ID NO: 15	SEQ ID NO: 16	SEQ ID NO: 17	SEQ ID NO: 18

Set	F3	B3	FIP	BIP	LF	LB
Set-7	SEQ ID NO: 27	SEQ ID NO: 28	SEQ ID NO: 29	SEQ ID NO: 30	SEQ ID NO: 31	SEQ ID NO: 32
Set-8	SEQ ID NO: 19	SEQ ID NO: 33	SEQ ID NO: 21	SEQ ID NO: 22	SEQ ID NO: 23	SEQ ID NO: 34
Set-9	SEQ ID NO: 35	SEQ ID NO: 36	SEQ ID NO: 37	SEQ ID NO: 38	SEQ ID NO: 39	SEQ ID NO: 40
Set-10	SEQ ID NO: 13	SEQ ID NO: 14	SEQ ID NO: 72	SEQ ID NO: 16	SEQ ID NO: 41	SEQ ID NO: 18
Set-11	SEQ ID NO: 42	SEQ ID NO: 43	SEQ ID NO: 44	SEQ ID NO: 45	SEQ ID NO: 46	SEQ ID NO: 47
Set-12	SEQ ID NO: 48	SEQ ID NO: 49	SEQ ID NO: 50	SEQ ID NO: 51	SEQ ID NO: 52	SEQ ID NO: 53
Set-13	SEQ ID NO: 54	SEQ ID NO: 55	SEQ ID NO: 56	SEQ ID NO: 57	SEQ ID NO: 58	SEQ ID NO: 59
Set-14	SEQ ID NO: 64	SEQ ID NO: 65	SEQ ID NO: 60	SEQ ID NO: 61	SEQ ID NO: 62	SEQ ID NO: 63
Set-15	SEQ ID NO: 70	SEQ ID NO: 71	SEQ ID NO: 66	SEQ ID NO: 67	SEQ ID NO: 68	SEQ ID NO: 69

[0052] Typically, 3 to 5 µL of extracted nucleic acid material or negative controls (NU = negative urine; NTC = nuclease free water or Tris buffer, no template control) served as template for RT-LAMP reactions. 10-25 µl total volume reactions were prepared on ice as mixes containing formulations including 1x amplification buffer comprising 10-40 mM Tris-HCl, 0-0.5% Tween 20, 0-300mM Trehalose, 5-70 mM KCl, 4-10 mM MgSO4, 10-20 mM (NH4)2SO4, 0-2mM TCEP and 1.6-2mM each dCTP, dGTP, dATP and dTTP. NEB Bst2 polymerase (NEB CN# M0537L) and RTx Warmstart reverse transcriptase (NEB CN# M0380S) enzymes. Primers (2 µM inner primers, 0.2 µM outer primers, and 0.8 µM Loop primers) were added to individual reactions or directly to master mixes as required per experimental design. Molecular beacons (0.2 µM) or 200 nM Yo-Pro-1, Yo-Pro-3 or To-Pro dye was also added to the master mix, as indicated in the examples below. Amplification reactions were prepared with the standard 6-primer mix or a 7-primer mix where indicated (Sets 16-27). Master mixes were distributed to individual sample templates, vortexed and centrifuged briefly and each reaction loaded into individual wells of a 96- or 384-well plate (Roche CN# 4729692001 or BioRad CNhsI9605). Reactions were carried out at temperatures ranging from 60-67°C and fluorescence monitored on either a Roche LightCycler 96 Real-Time PCR instrument or a BioRad CFX96 real time cycler. Target amplification was monitored via intercalating dye or molecular beacon probe binding to target resulting in release of molecular beacon fluorescence intramolecular quenching.

Example 2: LAMP with Dye Detection

[0053] A negative urine matrix was spiked with titred *N. gonorrhoeae* (serially diluted in PBS, Zeptometrix CN # 0801482, ATCC CN#19424 or ATCC CN#49226). Nucleic acids were extracted from the spiked sample using standard extraction methods and the sample was amplified using LAMP primers (as described in Table 2). YoPro™ dye or a compatible wavelength version within the same dye set family (Life Technologies; green fluorescent carbocyanine nucleic acid stain) was used for the detection of the amplified product. The master mix was prepared as described in Example 1. Results are summarized in Table 3. NT indicates conditions not tested. “No Amp” indicates that no amplification was detected.

TABLE 3 - Time to Positive (Dye Detection)

Set	5x10^{3}CFU/mL	5 CFU/mL	NTC	NU
Set-12	12.89	21.66	No Amp	No Amp
Set-13	6.28±0.18	9.22±0.26	42.2 (1 of 2)	No Amp
Set-14	7.55	10.93	29.03	34.98
Set-15	4.68	6.88	39.71	42.84
Set-2	5.5±0.099	7.94±0.13	26.36±1.46	45.18±1.95
Set-3	9.47±0.11	13.64±0.37	No Amp	No Amp
Set-4	5.90±0.03	8.63±0.20	33.09±5.23	34.87±4.18
Set-5	5.15±0.15	7.57±0.16	29.72±10.81	24.31±4.96
Set-6	9.58±0.01	14.10±1.00	No Amp	No Amp
Set-7	6.59±0.01	9.42±0.09	No Amp	35.84±3.22
Set-8	5.26±0.19	7.57±0.01	36.17±1.19	29.5±1.53
Set-1	12.56±0.94* (*5x10^2 CFU/mL)	No Amp	No Amp	NT
Set-9	4.25 (*5x10^2 CFU/mL)	5.00±0.68	37.31 (1 of 3)	NT
Set-10	7.42 (*5x10^2 CFU/mL)	11.18±0.25	No Amp	NT
Set-11	4.90 (*5x10^2 CFU/mL)	5.92±1.15	25.522.15 (3 of 3)	NT

Example 3: Specificity

[0054] A subset of the primer sets described in Example 2 were additionally tested for specificity by comparing reactions with 5x10^3 and 5 CFU/mL of extracted *N. gonorrhoeae* nucleic acid template (NG) to reactions with 5x10^5-1x10^6 CFU/mL of extracted nucleic acid template from closely related *Neisseria* species (live titred stocks were purchased from Zeptometrix), *Neisseria meningitides* (NM), *Neisseria lactamica* (NL), and *Neisseria sicca* (NS), negative urine extractions, or no template controls (NTC). When the amplification reactions were performed as described in Example 1, a number of the primer sets tested demonstrated some level of cross-reactivity against additional *Neisseria* species (Table 4).

TABLE 4 - Cross-Reactivity (Dye Detection)

Set	T_p NG 5x10^3 CFU/mL	NL	NS	NM	NTC	Neg Urine
Set-12	12.89	No Amp	No Amp	No Amp	No Amp	No Amp
Set-13	6.28±0.18	No Amp	No Amp	No Amp	42.2 (1 of 2)	No Amp
Set-14	7.55	30.88	37.03	No call	29.03	34.98
Set-15	4.68	35.38	49.98	47.75	39.71	42.84
Set-2	5.5±0.099	29.87 ± 13.72	44.95±3.75	28.41±11.02	26.36±1.46	45.18±1.95
Set-3	9.47±0.11	No Amp	No Amp	No Amp	No Amp	No Amp
Set-4	5.90±0.03	18.46 ± 4.28	18.53 ± 7.46	18.48 ± 7.55	33.09±5.23	34.87±4.18
Set-5	5.15±0.15	34.5±0.55	22.10±2.38	35.98±9.96	29.72±10.81	24.31±4.96
Set-6	9.58±0.01	No Amp	No Amp	No Amp	No Amp	No Amp
Set-7	6.59±0.01	26.3 (1 of 2)	No Amp	No Amp	No Amp	35.84±3.22
Set-8	5.26±0.19	30.18±3.00	30.45±3.97	33.53±5.54	36.17±1.19	29.5±1.53
Set-1	12.56±0.94* (*5x10^2 CFU/mL)	No Amp	No Amp	24.33±0.52	No Amp	NT
Set-9	4.25 (*5x10^2 CFU/mL)	NT	NT	NT	37.31 (1 of 3)	NT
Set-10	7.42 (*5x10^2 CFU/mL)	NT	NT	NT	No Amp	NT
Set-11	4.90 (*5x10^2 CFU/mL)	NT	NT	NT	25.522.15 (3 of 3)	NT

Example 4: Molecular Beacon Detection

[0055] To provide an additional level of direct sequence-based detection of amplified product (as opposed to indirect dye detection), molecular beacons, MB1 to MB4 (SEQ ID NOs: 73-76, respectively) targeting unique nucleotides within the *N. gonorrhoeae* amplicon of primer sets with promising Tp's combined with sensitivity, were designed and utilized for detection of amplification from nucleic acid extracted from live bacteria (Table 5). The molecular beacon probe was designed with 5' fluorophore/3' quencher modifications (6-Carboxyfluorescein (FAM)) and Black Hole Quencher 1 (BHQ1) included to provide target-specific fluorescent detection.

TABLE 5 - Probe Sequences

ID	Fluor	Quench	Sequence (5′ to 3′)	Sequence ID

MB1	FAM	BHQ1	CCGCA**ACGTGCCGCCGATGTTG**CGG	SEQ ID NO: 73
MB2	FAM	BHQ1	GCGT**GACGGACGTGCCGCCGATGT**CACGC	SEQ ID NO: 74
MB3	FAM	BHQ1	CG**TTGGGACGGACGTGCCGCC**AACG	SEQ ID NO: 75
MB4	FAM	BHQ1	CG**GGGACGGACGTGCCGCCGATGT**CCCCG	SEQ ID NO: 76

[0056] 10-25 µl total volume reactions were evaluated utilizing eluate from 5 to 5x10^3 CFU/mL extractions of *N. gonorrhoeae* as template input according to the methods described in Example 1. While use of a Molecular Beacon for detection resulted in a slight increase in reaction Tp, the ability to directly detect amplification products based on sequence, and thereby distinguish closely related species, is a desirable result.

TABLE 6 - Time to Positive Cross-Reactivity (Probe Detection)

Primers	Beacon	5x10^3 CFU/mL	5 CFU/mL	NL	NS	NM	NC	NU	NTC
Set-13	MB1	9.49	13.61	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
Set-13	MB2	8.42	12.01	NT	NT	NT	NT	NT	No Amp
Set-13	MB3	8.82±0.71	12.54±0.81	No Amp	No Amp	No Amp	19.8	No Amp	No Amp
Set-13	MB4	8.25±0.89	11.72±0.60	No Amp	No Amp	No Amp	22.01	No Amp	No Amp
Set-14	MB1	10.55±0.03	14.47±0.39	No Amp	No Amp	No Amp	21.32	No Amp	No Amp
Set-14	MB2	12.44±0.04	16.94±0.11	No Amp	No Amp	No Amp	25.14	No Amp	No Amp
Set-14	MB3	10.95±0.16	14.71	No Amp	No Amp	No Amp	23.04	No Amp	No Amp
Set-14	MB4	11.15±0.06	14.94±0.03	No Amp	No Amp	No Amp	24.01	No Amp	No Amp
Set-15	MB1	4.74±0.60	7.76±0.73	No Amp	No Amp	No Amp	18.16	13.48	No Amp
Set-15	MB2	8.09±0.06	11.26±0.11	No Amp	No Amp	No Amp	31.33	No Amp	No Amp
Set-15	MB3	7.43±0.12	10.18±0.47	No Amp	No Amp	No Amp	24.17	32.86	No Amp
Set-15	MB4	7.97±0.13	10.4±0.10	35.23	No Amp	No Amp	27.53	No Amp	No Amp

[0057] 25 µl total volume reactions were prepared with eluate from extraction for 3 CFU/mL of *Neisseria gonorrhoeae* as template input. Reactions were performed according to the methods described in Example 1.

TABLE 7 - Time to Positive (Probe Detection)

Primers	Beacon	3 CFU/mL	Freq. of Positive	NTC
Set-13	SEQ ID NO: 75	18.76±2.34	67%	No Amp
Set-15	SEQ ID NO: 75	18.28±0.21	67%	No Amp

Example 5: Extended Bench Sensitivity Evaluation

[0058] A urine matrix pool from multiple anonymous donors was screened to confirm a *N. gonorrhoeae* negative status, aliquoted and stored at -80°C. *N. gonorrhoeae* (ATCC CN#19424) were serially diluted in Mueller Hinton media and thawed pooled urine aliquots spiked with N. gonorrhoeae to a final concentration of 10X, 3X, 1X, 0.5X or 0.25X of the assay sensitivity as estimated via bench extraction and wet bench RT-LAMP. Samples were prepared with or without inclusion of a stabilizing solution. A Negative urine control with or without stabilizing solution was also included. See Table 8 for specific sample and stabilizer volume inputs.

[0059] Nucleic acids were extracted from the spiked sample using standard extraction methods and the sample was amplified using LAMP primers Set-15, as described in Example 2. Molecular beacon, SEQ ID NO. 75, was used for the detection of the amplified product. The master mix was prepared as described in Example 1. Results are summarized in Table 9. NT indicates conditions not tested. “No Amp” indicates that no amplification was detected.

TABLE 8 – Volumes of Urine and Stabilizer Used For Sample Preparation

	Sample Preparation		Testing
Sample	Stabilizer (mL)	Spiked urine (mL)	Processed Vol. (mL)
neat urine, no stabilizer	0	1	1
urine + stabilizer	3	6	1

TABLE 9 – Time to Positive (Probe Detection)

Conc. (CFU/mL)	Stabilizer? Y/N	Tp (min.)	Freq. of Positive

2	N	8.43±0.37	20/20
0.6		8.79±0.29	45/45
0.2		9.80±1.16	38/45
0.1		10.51±1.31	45/45
0.05		11.08±1.11	43/45
0		No Amp	0/5

[0060] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications can be made thereto without departing from the spirit or scope of the appended claims. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0061] Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

We claim:

1. A composition comprising a sequence-specific primer set selected from the group consisting of Set-1 through Set-15.

2. The composition of claim 1, wherein the sequence-specific primer set is selected from the group consisting of: Set-5, Set-11, Set-13, Set-14 and Set-15.

3. The method of claim 2, wherein the sequence-specific primer set is Set-15.

4. The composition of claim 1, further comprising a probe.

5. The composition of claim 4, wherein the probe comprises a label.

6. The composition of claim 5, wherein the probe is a labeled polynucleotide.

7. The composition of claim 6, wherein the labeled polynucleotide comprises a sequence selected from the group consisting of: nucleotides 6-22 of SEQ ID NO: 73, nucleotides 5-24- of SEQ ID NO: 74, nucleotides 3-21 of SEQ ID NO: 75, and nucleotides 3-24 of SEQ ID NO: 76.

8. The composition of claim 6, wherein the labeled polynucleotide comprises a sequence selected from the group consisting of SEQ ID NO: 73 through SEQ ID NO: 76.

9. The composition of claim 6, wherein the label is a fluorophore.

10. The composition of claim 9, wherein the fluorophore is covalently attached to a terminus of the polynucleotide.

11. The composition of claim 4, wherein the probe is a molecular beacon comprising a fluorophore, a quencher, and a polynucleotide.

12. The composition of claim 11, wherein the molecular beacon comprises a sequence selected from the group consisting of SEQ ID NO: 73 through SEQ ID NO: 76.

13. The composition of claim 12, wherein the polynucleotide sequence consists of SEQ ID NO: 75.

14. A method of detecting *Neisseria gonorrhoeae* in a test sample, the method comprising:

extracting nucleic acid from the test sample;

amplifying a target sequence by reacting the nucleic acid extracted in step (a) with a reaction mixture comprising a strand displacement DNA polymerase and a sequence-specific primer set, wherein said sequence-specific primer set is selected from the group consisting of Set-1 through Set-15; and

detecting the presence or absence of an amplified product of step (b), wherein the presence of said amplification product is indicative of the presence of *Neisseria Gonorrhoeae* in the test sample.

15. The method of claim 14, wherein the amplification step is performed for less than 15 minutes.

16. The method of claim 14, wherein the amplification step is performed for less than ten minutes.

17. The method of claim 14, wherein the reaction mixture further comprises a reverse transcriptase.

18. The method of claim 14, wherein detecting the presence or absence of the amplification product comprises hybridizing the amplified product with a probe comprising a polynucleotide attached to a label.

19. The method of claim 18, wherein the labeled polynucleotide comprises a sequence selected from the group consisting of: nucleotides 6-22 of SEQ ID NO: 73, nucleotides 5-24- of SEQ ID NO: 74, nucleotides 3-21 of SEQ ID NO: 75, and nucleotides 3-24 of SEQ ID NO: 76.

20. The method of claim 19, wherein the labeled polynucleotide comprises a sequence selected from the group consisting of: SEQ ID NO: 73 through SEQ ID NO: 76.

21. The method of claim 14, wherein the sequence-specific primer set is selected from the group consisting of: Set-5, Set-11, Set-13, Set-14 and Set-15.

22. The method of claim 21, wherein the sequence-specific primer set is Set-15.

23. The method of claim 14, wherein *Neisseria Gonorrhoeae* is present in the test sample at a concentration of ≤ 100 CFU/mL.

24. The method of claim 23, wherein *Neisseria Gonorrhoeae* is present in a test sample at a concentration of ≤ 10 CFU/mL.

25. The method of claim 24, wherein *Neisseria Gonorrhoeae* is present in the test sample at a concentration of ≤ 10 CFU/mL and the amplification reaction is performed for less than 15 minutes.

26. A kit comprising a composition according to claim 1.

27. The kit of claim 26, further comprising a strand displacement polymerase.

28. The kit of claim 27, further comprising a reverse transcriptase.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/43620

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07H 21/04, C07K 14/22, A61P 31/04, C12Q 1/68 (2020.01)

CPC - C12Q 1/689, C12Q 2600/16, Y10S 435/871, Y10S 435/822

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2016/0024562 A1 (ABBOTT MOLECULAR INC.) 28 January 2016 (28.01.2016) Abstract; Claim 5; Claim 9	1, 4-12, 26-28
A	WO 2002/079243 A2 (CHIRON SPA et al.) 10 October 2002 (10.10.2002) p341, SEQ ID 4451; p343, SEQ ID 4475	1, 4-12, 26-28
A	GenBank submission LR606187.1, Aquila chrysaetos chrysaetos genome assembly, chromosome: 7, 04 July 2019 [online]. [Retrieved on 23 November 2020]. Retrieved from the internet: <URL: https://www.ncbi.nlm.nih.gov/nuccore/LR606187>, Entire document	1, 4-12, 26-28
A	GenBank submission EK565433.1, 1095521038908 Global-Ocean-Sampling_GS-32-01-01-1P3-1P6KB marine metagenome genomic clone 1061005966854 5', genomic survey sequence, 26 May 2010 [online]. [Retrieved on 23 November 2020]. Retrieved from the internet: <URL: https://www.ncbi.nlm.nih.gov/nuccore/EK565433>, Entire document	1, 4-12, 26-28
A	GenBank submission LS483369.1, Neisseria cinerea strain NCTC10294 genome assembly, chromosome: 1, 17 June 2018 [online]. [Retrieved on 23 November 2020]. Retrieved from the internet: <URL: https://www.ncbi.nlm.nih.gov/nuccore/LS483369>, Entire document	1, 4-12, 26-28
A	GenBank submission AE004969.1, Neisseria gonorrhoeae FA 1090, complete genome, 1 July 2015 [online]. [Retrieved on 23 November 2020]. Retrieved from the internet: <URL: https://www.ncbi.nlm.nih.gov/nuccore/AE004969>, Entire document	1, 4-12, 26-28

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

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"D" document cited by the applicant in the international application	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search 23 November 2020	Date of mailing of the international search report 31 DEC 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.	PCT/US 20/43620

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
----Please see continuation in first extra sheet ----------------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 4-12, 26-28, limited to primer Set-1 (SEQ ID NOs: 1-6) and probe of SEQ ID NO:73

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of Box No. III. Observations where unity of invention is lacking.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I+, Claims 1-13, 26-28, directed to a composition comprising a sequence-specific primer set. The composition will be searched to the extent that the composition encompasses a primer set consisting of Set-1 comprising SEQ ID Nos:1-6, and a probe wherein the probe comprises nucleotides 6-22 of SEQ ID NO: 73 (note, these are the first claimed sequences for a primer set and probe of the inventive composition). It is believed that claims 1, 4-12, 26-28 encompass this first named invention, and thus these claims will be searched without fee to the extent that the composition encompasses a primer set consisting of Set-1 comprising SEQ ID Nos:1-6 and a probe wherein the probe comprises nucleotides 6-22 of SEQ ID NO: 73. Additional composition(s) comprising additional primer set(s) and/or probe sequence(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected composition(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a composition comprising a primer set consisting of Set-2 comprising SEQ ID Nos:7-12 and a probe wherein the probe comprises nucleotides 6-22 of SEQ ID NO: 73 (claims 1, 4-12, 26-28).

Group II+, Claims 14-25, directed to a method of detecting Neisseria gonorrhoeae in a test sample. Group II+ will be searched upon payment of additional fees. The method may be searched, for example, to encompass a sequence-specific primer set of Set-1 comprising SEQ ID Nos:1-6 and a probe wherein the probe comprises nucleotides 6-22 of SEQ ID NO: 73 for an additional fee and election as such. It is believed that claims 14-20, 23-25 read on this exemplary invention. Additional method(s) comprising additional primer set(s) and/or probe sequence(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected method(s) comprising additional primer set(s) and/or probe sequence(s). Failure to clearly identify how any paid additional invention fees are to be applied to the"+" group(s) will result in only the first claimed invention to be searched. Another exemplary election would be a method comprising a primer set consisting of Set-2 comprising SEQ ID Nos:7-12 and a probe wherein the probe comprises nucleotides 6-22 of SEQ ID NO: 73 (claims 14-20, 23-25).

The inventions listed as Group I+ and Group II+ do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features
The inventions of Group I+ and Group II+ each include the special technical feature of a unique nucleic acid sequence. Each nucleic acid sequence comprises a unique probe, and is considered a distinct technical feature.
Additionally, Group I+ has the special technical feature of a primer set composition, that is not required by Group II+.
Group II+ has the special technical feature of a detection method, that is not required by Group I+.

Common technical features

No technical features are shared between the primers and/or probe nucleic acid sequences in each of Groups I+ and II+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ and II+ were considered to share the technical features of including:
a sequence-specific primer set, these shared technical features are previously disclosed by prior art, as discussed below.

Group II+ inventions additionally share the technical feature of:
method of detecting Neisseria gonorrhoeae in a test sample, the method comprising:
extracting nucleic acid from the test sample;
amplifying a target sequence by reacting the nucleic acid extracted in step (a) with a reaction mixture comprising a strand displacement DNA polymerase and a sequence-specific primer set, wherein said sequence-specific primer set is selected from the group consisting of Set-1 through Set-15; and
detecting the presence or absence of an amplified product of step (b), wherein the presence of said amplification product is indicative of the presence of Neisseria Gonorrhoeae in the test sample. However, these shared technical features are previously disclosed by US 2016/0024562 A1 to Abbott Molecular Inc., (hereinafter 'Abbott').

-------please see continuation on next sheet---------------------

Continuation of Box No. III. Observations where unity of invention is lacking.

-----------continued from previous sheet----------------------

Abbott teaches a method of detecting Neisseria gonorrhoeae in a test sample (Abstract - 'Polynucleotides useful for detecting Chlamydia trachomatis and/or Neisseria gonorrhoeae in a test sample, kits, a nucleic acid amplification method and detection method including the same.'; Claim 9 - 'A method of amplifying Chlamydia trachomatis and/or Neisseria gonorrhoeae in a test sample'), the method comprising:
amplifying a target sequence by reacting the nucleic acid extracted in step (a) with a reaction mixture comprising a DNA polymerase and a sequence-specific primer set (Claim 9 - 'said method comprising: (a) forming a reaction mixture comprising nucleic acid amplification reagents, a test sample potentially containing a Chlamydia trachomatis and/or Neisseria gonorrhoeae target sequence a composition according to claim 1; and(b) subjecting the mixture to amplification conditions to generate at least one copy of a nucleic acid sequence complementary to the target sequence.'; para [0114] - 'PCR was performed in 1x GeneAmp PCR Gold Buffer, 0.25 mM EDTA and 0.125 mM EGTA. Amplitaq Gold DNA polymerase was used'; Claim 5 - 'A primer/probe set selected from the primer/probe sets consisting of: Primer and Probe Set 1 (SEQ ID NOs: 1, 4, and 5);'), and detecting the presence or absence of an amplified product of step (b), wherein the presence of said amplification product is indicative of the presence of Neisseria Gonorrhoeae in the test sample (Claim 11 - '(c) hybridizing the probe to the nucleic acid sequence complementary to the target sequence, so as to form a hybrid comprising the probe and the nucleic acid sequence complementary to the target sequence; and (d) detecting the hybrid as an indication of the presence of Chlamydia trachomatis and/or Neisseria gonorrhoeae in the test sample.'). Abbott does not expressly teach extracting nucleic acid from the test sample, or use of a strand displacement DNA polymerase. However, since Abbott teaches use of SDA (para [0036] - 'Amplification procedures are well-known in the art and include, but are not limited to, polymerase chain reaction (PCR), TMA, rolling circle amplification, nucleic acid sequence based amplification (NASBA), and strand displacement amplification (SDA).'), it would have been obvious to one of ordinary skill in the art that a strand displacement DNA polymerase could be used for the strand displacement amplification. Further, it would have been obvious that nucleic acid could be extracted from the test sample using routine procedures, to provide polynucleotide substrate for the PCR test.

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I+ and II+ inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.

NOTE, Claim 3 drafted as dependent of the method of claim 2 is objected to for improper antecedent because claim 2 recites a composition, not a method. For this application, claim 3 is reconstrued as though dependent on the composition of claim 2.