



**NUI Galway**  
**OÉ Gaillimh**

**Novel Multiplex Isothermal Nucleic Acid  
Amplification Technologies for the Detection of  
Bacterial Meningitis Associated Pathogens**

A thesis submitted to the National University of Ireland, Galway, for the  
degree of Doctor of Philosophy

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## **Abstract**

Infectious diseases, caused by pathogenic microorganisms such as bacteria, fungi or viruses, are a leading cause of significant global morbidity and mortality, with over 10 million associated deaths reported each year. Respiratory tract infections, such as bacterial meningitis, account for the highest rates of infectious disease related mortality. Bacterial meningitis infection is caused by human commensal bacteria that invade the respiratory tract and central nervous system leading to inflammation and rapid onset of symptoms, which can be fatal if untreated. *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* are the most common etiological agents of bacterial meningitis infection. *S. pneumoniae* and *N. meningitidis* are responsible for over 80% of all bacterial meningitis cases since the implementation of the *H. influenzae* type b (Hib) conjugate vaccine. However, *H. influenzae* related meningitis remains a serious threat in regions without immunisation and especially amongst children under five years of age. Global vaccination programmes have significantly reduced the occurrence of bacterial meningitis infection, however, approximately 1.2 million cases and over 100,000 related deaths are still reported annually, with increasing incidents of drug resistance leading to treatment failures. The global burden of bacterial meningitis infection is directly related to socioeconomic factors, with the highest rates of infection in low-resourced developing regions. The rate of infection in developed regions is approximately 1 in 100,000 persons per year, however, this can be as high as 20-40 in 100,000 persons per year in developing regions, with significantly higher mortality rates and severe impairments in survivors. The rapid and accurate detection of bacterial meningitis pathogens is essential for early and appropriate treatment which is directly linked with lower morbidity and mortality rates, and prevents broad-range antibiotic administration, a major contributing factor to antimicrobial-resistance dissemination. Isothermal nucleic acid amplification technologies such as recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP) offer rapid, sensitive and specific diagnostic approaches for the identification of bacterial meningitis associated pathogens. Additionally, these methods are low-cost, easy-to-use and compatible with point-of-care (POC) diagnostic technologies, essential for near-patient testing in poorer disease-burdened regions.

This thesis describes the development and evaluation of novel RPA and LAMP technologies for the detection of *S. pneumoniae*, *N. meningitidis* and *H. influenzae*. These technologies include: three internally controlled duplex RPA assays for the detection of each pathogen; a novel *Tth* endonuclease cleavage loop-mediated isothermal amplification (TEC-LAMP) assay for the internally controlled multiplex detection of these pathogens; and novel loop-primer endonuclease cleavage loop-mediated isothermal amplification (LEC-LAMP) technology for the singleplex or multiplex detection of each pathogen with flexible single-base specificity for effective single nucleotide polymorphism detection. All technologies demonstrated efficient analytical specificity when tested with extensive exclusivity and inclusivity bacterial reference strains, and low limits of detection using Probit regression analysis. Clinical testing of each technology, using samples from confirmed cases of bacterial meningitis infection, demonstrated efficient diagnostic specificity and sensitivity. This thesis advanced the current state-of-the-art in multiplex isothermal nucleic acid amplification detection of these major bacterial meningitis pathogens, providing novel transferable diagnostics technology for infectious disease POC testing.

**Chapter 1**  
**Introduction**

## 1.1 Infectious diseases and diagnosis

Physiological impairment of an organism, as a whole or at a localised area, that produces specific symptoms which are not a result of physical injury, is referred to as a “disease”. The causative factors leading to the occurrence of a disease can be either intrinsic or extrinsic in nature. Intrinsic causative factors originate from within an organism, usually as a result of genetic predisposition, while extrinsic causative factors are a result of an organisms interaction with external agents. Diseases that originate from extrinsic factors, such as the invasion of an organism by a foreign agent whose activities prevent standard host functioning, are referred to as infectious diseases [1, 2]. Microorganisms such as bacteria, fungi, viruses and parasites are the typical extrinsic factors that cause infectious diseases in humans. Zoonotic infectious diseases are caused by the transmission of pathogenic microorganisms from animals to humans. The type and severity of impairment caused to the host by a particular invading microorganism, in combination with the pathogens method of entry, is used to categorise infectious diseases. Infection initiates with entry of the causative agent into the host via locations such as the skin, mouth, eyes, nose and genitals. Localised colonisation and avoidance of the hosts defence mechanisms leads to multiplication of the invading pathogen followed by release of harmful metabolic products causing damage to the host which can be fatal if untreated [3].

Infectious diseases are a leading cause of morbidity and mortality throughout the world, especially in low-resource developing countries [4, 5]. In 2016, over 10 million deaths were related to infectious diseases, accounting for approximately one-fifth of total global mortality. Of these infectious disease related deaths, lower respiratory tract infections accounted for the highest mortality rates, followed by enteric infections, tuberculosis, human immunodeficiency virus (HIV) linked with acquired immunodeficiency syndrome (AIDS), and malaria [6]. Additionally, various other emerging and re-emerging infectious diseases cause major global public health problems, in particular, influenza, dengue virus, severe acute respiratory syndrome (SARS), Ebola virus disease (EVD), Middle East respiratory syndrome (MERS) and Zika virus [7-12]. The associated morbidity and disability caused by these infectious diseases results in significant negative impact on global public health which is commonly quantified in disability-adjusted life years (DALYs) [13, 14]. The global burden of infectious diseases and the associated rate of occurrence is directly related

to socioeconomic factors, with the highest rates of infection in poorer developing regions. Diseases that are typically characterised by high morbidity and mortality rates are most prevalent in these low-resourced areas with poor infrastructure and vulnerable populations [15, 16].

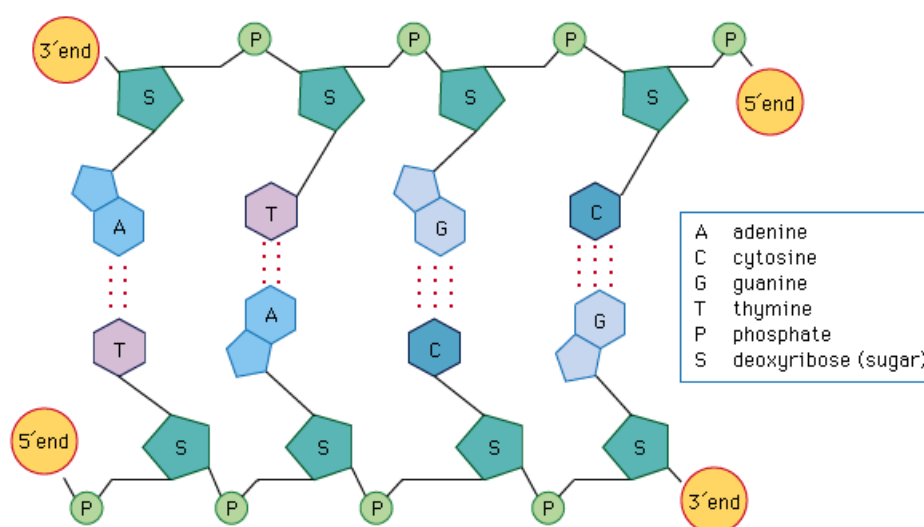
Diagnosis and treatment of infectious diseases involves initial physical examination for classic symptoms, followed by administration of general antibiotics, antiviral, antifungal or antiparasitic agents [17, 18]. The effectiveness of such treatments, however, is directly dependant on timely and accurate diagnosis, with laboratory diagnostics typically required for determination of the causative pathogens [4]. Conventional laboratory diagnostics of infectious diseases involves the culturing of blood and tissue samples using methodologies such as Gram-staining, microscopy and biochemical methods such as enzyme immunoassays and agglutination. These methods however can be time consuming, labour intensive and are often unreliable in cases of prior treatment [4]. Molecular diagnostic methods such as microarrays, sequencing and nucleic acid amplification, target genomic sequence information relating to infectious disease associated pathogens. These methods offer improved infectious disease diagnostic alternatives over conventional methods in terms of sensitivity, specificity and time-to-detection. As a means of combating the threat of infectious diseases, especially in the most disease-prevalent low-resourced regions, there is a focus on developing technologies that combine these molecular diagnostic methods with point-of-care (POC) devices [19].

POC infectious disease diagnostics involves the performance of near-patient sample analysis outside laboratory settings using miniaturised, transportable, analytical devices that produce real-time results enabling fast and accurate treatment. POC diagnostic tests are designed as small, easy-to-use, plastic chip cassettes or membrane-based test units. These devices typically process patient samples through a combination of microfluidic methods and nucleic acid amplification technologies. The various benefits of this diagnostic approach includes low cost, fast analysis turnaround time, automation and high throughput ability [20]. The World Health Organisation ASSURED criteria is a set of benchmarking guidelines stating the ideal characteristics of fully integrated POC diagnostic technologies for application in resource-limited settings. These ASSURED characteristics state that POC technologies should be affordable, sensitive, specific, user-friendly, rapid, equipment free and deliverable

[21]. The mobilisation of POC technologies utilising effective diagnostic methods such as nucleic acid amplification can lead to early intervention, prevention of disease dissemination, improved disease surveillance and reduced threat of epidemics or pandemics.

## 1.2 Nucleic acid amplification diagnostics

Nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are macromolecule biopolymers found in all living organisms that are essential for all forms of life. DNA and RNA are composed of nucleotide monomers that consist of carbon sugars bound to a phosphate group which are coordinated to different nitrogenous bases. These nitrogenous bases are either pyrimidine, consisting of cytosine, thymine or uracil; or purine, consisting of adenine or guanine. Nucleotide monomers form nucleic acid strands with a backbone of alternating sugars and phosphates creating a unique sequence of nitrogenous bases. Nucleic acid strands have 5'-ends consisting of a phosphate group attached to a 5' sugar carbon, and 3'-ends consisting of a free hydroxyl group attached to a 3' sugar carbon, resulting in either a 5'-3' or 3'-5' directionality. Addition of nucleotides to these strands occurs at the free hydroxyl group 3'-end. DNA consists of two nucleic acid strands in a double helix structure (Figure 1.1) that are antiparallel, enabling complementary nitrogenous base pairing via hydrogen bonds [22].



**Figure 1.1: DNA structure [23].**

Complementary base pairing, discovered by Watson and Crick in 1953 [24], is the unique and specific pairing that exists between guanine and cytosine, and adenine and thymine. RNA is a single-stranded nucleic acid that contains uracil in place of thymine. Hereditary information for all living organisms is stored in the specific nucleotide sequences of DNA and RNA. The Central Dogma in molecular biology involves the RNA polymerase-mediated transcription of DNA into messenger RNA (mRNA), followed by the ribosomal-mediated translation of this mRNA into proteins responsible for cellular function [22]. Nucleic acid amplification diagnostics utilises the unique Watson-Crick complementary base pairing to enable the highly specific identification and differentiation of microorganisms.

Nucleic acid amplification diagnostics are platform methodologies used to generate multiple copies of specific genomic nucleic acid sequences associated with an organism for the purposes of identification. Infectious disease diagnostics routinely utilise nucleic acid amplification technologies for the purposes of identifying particular bacteria, fungi or viruses in patient samples, enabling accurate diagnosis and treatment [25]. These methods can also identify single nucleotide sequence mutations or single nucleotide polymorphisms (SNPs) associated with particular microorganisms, to enable increased specificity or identification of antimicrobial resistances [26, 27]. The genomic nucleic acid sequences targeted by these methodologies are unique sequences known to be associated with particular microorganisms, and are commonly referred to as biomarkers or diagnostics targets. These biomarkers are usually highly conserved sequences in the genome of particular microorganisms, enabling identification and differentiation from closely related organisms. The most commonly used biomarkers in infectious disease diagnostics are the genes coding for prokaryotic 16S ribosomal RNA. These 16S rRNA genes are associated with a slow rate of evolution leading to consistent composition and high conservation in all known bacterial organisms [28-30].

Nucleic acid amplification diagnostics relies on the utilisation of genomic sequence information relating to all organisms stored on publically available databases. Primary databases, used to store original sequence data, belong to the International Nucleotide Sequence Database (INSD), consisting of the National Center for Biotechnology Information (GenBank, USA), the European Bioinformatics Institute (EMBL) and the National Institute of Genetics (DNA Data Bank of Japan). These databases act as

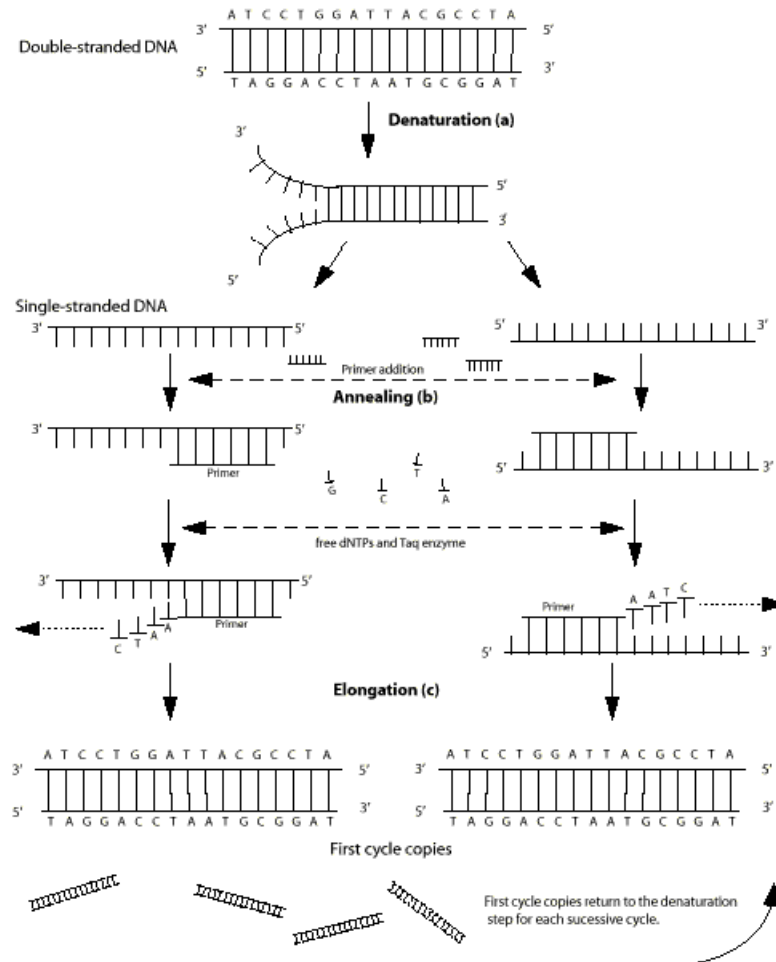
repositories for the submission of nucleic acid sequence information relating to all organisms. Collaboration between these databases ensures all data is accurate and updated regularly. The accumulation this genetic data has rapidly increased in recent years due to advances whole genome sequencing technologies [31]. This publically available information enables the effective application of various nucleic acid amplification diagnostics in the area of infectious disease diagnostics, with the gold standard of these method being real-time polymerase chain reaction (PCR).

### *1.2.1 Polymerase chain reaction (PCR)*

Polymerase chain reaction (PCR) is a thermocycling-mediated nucleic acid amplification technique used to target and amplify specific DNA sequences. It is the most widely used nucleic acid amplification method for the identification of infectious disease associated pathogens [32]. Conventional PCR was conceived by Kary Mullis in 1983, leading to the first PCR related publication detailing the novel enzymatic amplification of DNA by Saiki and colleagues in 1985 [33]. Following this, Mullis filed a patent relating to this novel technology [34] and eventually published his own work detailing this discovery [35]. The initial applications of PCR were impractical due to the requirement of thermolabile Klenow fragment additions to the reaction after temperature denaturing steps. In 1988, Saiki and colleagues reported a significant advancement to PCR technology with the incorporation of a thermostable *Taq* DNA polymerase from *Thermus aquaticus* [36]. The next major advance in PCR technology was the development of real-time reaction monitoring through the use of fluorescently labelled oligonucleotide hydrolysis probes [37, 38].

Standard PCR assays use forward and reverse single-stranded DNA (ssDNA) oligonucleotide primers to target and amplify DNA sequences. Various online tools can design PCR assays to target input sequences [39], however, primers can be designed manually using basic guidelines [40]. Typical PCR primer lengths are 16-24 bases long with dissociation temperatures ( $T_m$  values) of approximately 50-55°C, and guanine to cytosine ratios of 40-60%. Ideal amplicon size is usually 50-150 bases long, with smaller amplicon sizes enabling more efficient amplification. Basic PCR reactions incorporate the DNA template to be amplified, a thermostable DNA polymerase enzyme, magnesium sulphate enzyme cofactor, target specific

oligonucleotide primers, deoxyribonucleotide triphosphates (dNTPs) and reaction buffer, all in a single reaction tube. Thermocycling equipment which facilitates rapid heating and cooling is used to perform the PCR reaction (Figure 1.2).

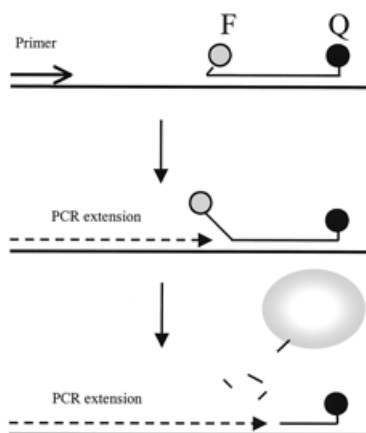


**Figure 1.2: PCR schematic overview [41].**

PCR reactions typically begin with an initial template denature phase of 95°C for 5 min, ensuring efficient separation and linearisation of the DNA which enables correct primer hybridisation prior to thermocycling. PCR cycling involves 30-40 cycles of: annealing, 60°C for 10-30 s; elongation, 72°C for 1-15 s; and denaturation, 95°C for 10-15 s. During the annealing phase, oligonucleotide primers bind to respective targets on the template DNA. Following this, the elongation phase causes DNA polymerase to bind to the free hydroxyl group on the 3'-end of the hybridised primers, initiating strand elongation with the addition of free dNTPs. After this, the denaturation phase causes the newly synthesised DNA strands to be separated, creating new primer target sites that enables the above process to be repeated. This repeated thermocycling results

in the exponential amplification of the target DNA sequence [42]. Reverse transcription PCR (RT-PCR) enables the amplification of RNA targets through incorporation of a reverse transcription enzyme directly into the PCR reaction [43]. Post-amplification gel electrophoresis using fluorescent DNA intercalating dyes and UV visualisation is typically used to analyse amplified PCR product. This post-amplification analysis, however, requires opening of the PCR reaction tubes leading to possible contamination issues [44].

Real-time PCR enables the real-time monitoring and quantification of PCR reactions through the use of platform instrumentation consisting of a thermal cycler, computerised optics for fluorescence acquisition and software for data analysis. There are two main approaches to the performance of real-time PCR, using fluorescent DNA intercalating dyes or fluorescently labelled oligonucleotide hydrolysis probes. Intercalating dyes, such as SYBR Green 1, fluoresce upon binding to double-stranded DNA (dsDNA) and the accumulation of amplified PCR product leads to a detectable fluorescence signal allowing for real-time amplification monitoring. Real-time PCR hydrolysis probes, such as TaqMan® (Figure 1.3), are fluorescently labelled oligonucleotides positioned between the forward and reverse PCR primers that enable fluorescence signal production at each PCR cycle [45]. Hydrolysis probes are 20-30 bases long with a higher  $T_m$  value than PCR primers of 60-65°C, and are coordinated with a 5'-end fluorophore and 3'-end quencher. The quencher label absorbs any fluorescence from the fluorophore and also acts as a polymerase extension blocker preventing any non-specific detection. The fluorophore label cannot be placed directly beside a guanine residue due to proximal G-base quenching and complementarity between the probe and primers should be avoided [46]. During real-time PCR, the hydrolysis probe will hybridise to the target before the primers due to its higher  $T_m$  value. Subsequent binding and polymerase extension from the primers during PCR will dissociate the hydrolysis probe due to the exonuclease activity of the polymerase enzyme, leading to fluorescence production. The level of fluorescence produced is relative to the amount of amplified product, allowing for real-time detection and quantification. Various forms of hybridisation probes are available to perform real-time PCR, such as, Scorpion® probes, molecular beacon probes and Locked Nucleic Acid (LNA®) probes. However, the Taqman® probe is one of the most commonly used hybridisation probes for real-time PCR diagnostics [47].



**Figure 1.3: TaqMan® hydrolysis probe.** Exonuclease activity of the DNA polymerase during PCR extension dissociates the quenched hydrolysis probe leading to fluorescence production [48].

Multiplex real-time PCR enables the simultaneous detection of multiple targets through the use of differentially labelled hydrolysis probes. Commonly used fluorophore labels in real-time PCR detection are FAM, ROX, HEX, CYAN and Cy5 dye labels. The fluorescence detection instrumentation used in real-time PCR has the capacity to detect different wavelengths produced by various fluorescent dyes permitting the simultaneous detection of multiple targets in one reaction [49, 50]. Multiplex detection is essential in nucleic acid diagnostics as it reduces analysis time and reagent cost, conserves clinical specimens and enables the incorporation of essential assay validating internal amplification controls (IACs) [51, 52]. An IAC involves the incorporation of a synthetic non-target template into an existing assay, which is either simultaneously co-amplified with the target DNA or detected in the absence of the target DNA. A negative result in a nucleic acid diagnostic assay can be a result of factors other than target absence, such as, reaction failure or inhibition, equipment malfunction, or operator error. Successful IAC detection in the event of no target detection confirms that a successful reaction was performed and validates the negative result. There are two main approaches for the use IAC's in a nucleic acid diagnostic assay, competitive or non-competitive. The competitive IAC approach uses one set of oligonucleotide primers to detect both the target and the IAC template, whereas the non-competitive approach uses two oligonucleotide primer sets, one set to detect the target DNA and the other to detect the IAC template. The IAC template is typically embedded into a vector system such as plasmid DNA for incorporation into the reaction. Various guidelines for the incorporation of IACs into nucleic acid

diagnostics have been established to improve standardisation for the emerging number of diagnostic technologies available today [52]. Additionally, MIQE guidelines are a set of industry standards detailing the minimum requirements for publications relating to quantitative real-time PCR assays [53].

Internally controlled multiplex real-time PCR technology provides sensitive and specific, validated, simultaneous multiple pathogen detection and has been extensively applied in the area of infectious disease diagnostics [54-56]. However, this technology is limited in its application in low-resourced highly disease-burdened regions. Real-time PCR requires skilled operation and expensive thermocycling equipment making it an impractical POC diagnostic option in these areas. Isothermal nucleic acid amplification methods provide comparable specificity and sensitivity to PCR without the requirement for thermocycling equipment, offering an improved alternative diagnostics option for poorer disease-prevalent regions.

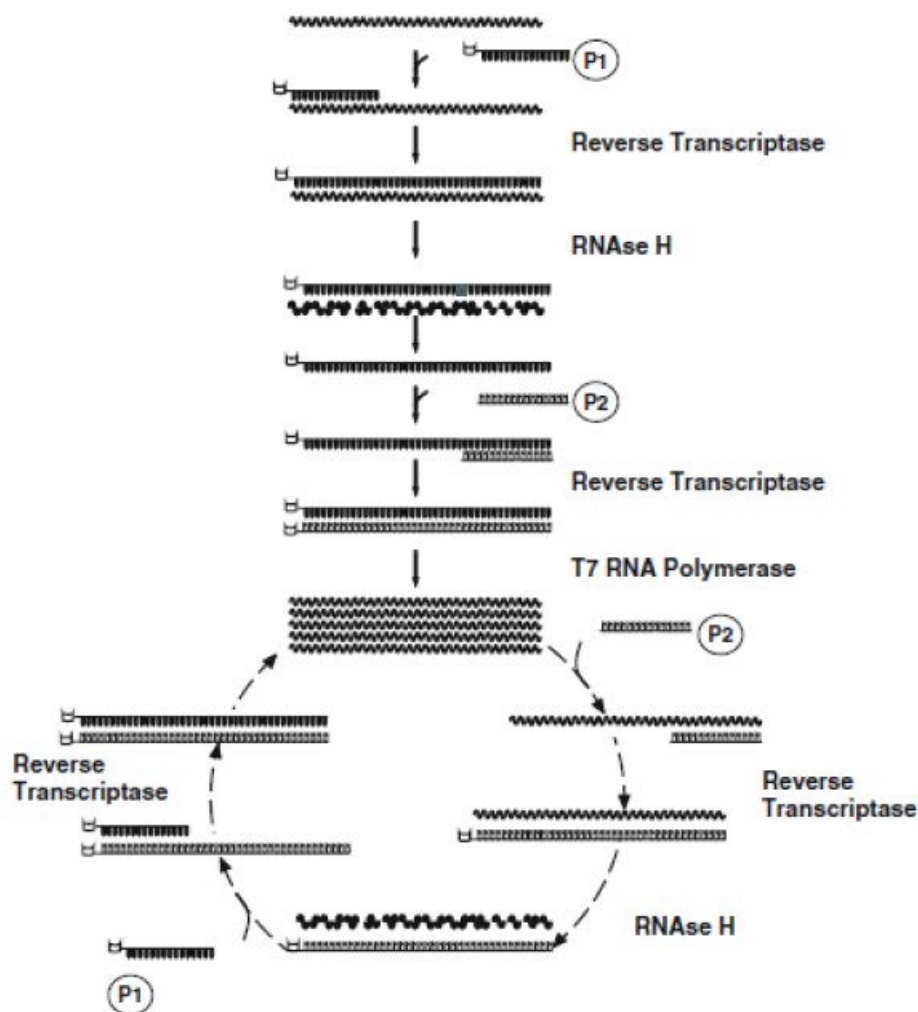
### **1.3 Isothermal nucleic acid amplification diagnostics**

Isothermal nucleic acid amplification technologies enable the accumulation of specific nucleic acid sequences for the identification of infectious disease associated pathogens using single-temperature reaction conditions. Unlike PCR, these technologies do not require complex thermocycling to enable denaturation, annealing and extension, and can be performed at low temperatures using basic equipment. Development of these technologies since the early 1990s has provided various simplified isothermal diagnostic alternatives to PCR, with each method differing by amplification process, monitoring methods and sample preparation. Most isothermal amplification methods are based on DNA replication processes, enzyme-based digestion or enzyme-free nucleic acid assembly. Typically, isothermal amplification methods utilise 1-3 enzymes such as polymerases, 2 or more oligonucleotide primers, are performed at temperature ranges from 40-60°C and take between 0.5-1 hours to complete. The most commonly used methods are strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA), helicase-dependent amplification (HDA), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA) and recombinase polymerase amplification (RPA). Advances in microfabrication has led to the incorporation of various isothermal amplification technologies with portable

microfluidics, capillary platforms and paper-based systems, enabling effective POC detection [57].

### 1.3.1 Nucleic acid sequence-based amplification (NASBA)

Nucleic acid sequence-based amplification (NASBA) is a transcription-mediated isothermal nucleic acid amplification method developed in 1991 by Compton and colleagues [58]. This technology, commonly referred to as self-sustained sequence replication (3SR), alternates the application of reverse transcription and DNA transcription to amplify RNA targets [59]. NASBA amplifies single-stranded RNA (ssRNA) in a similar process to that of retroviral RNA replication, utilising forward and reverse oligonucleotide primers in combination with three enzymes: avian myeloblastosis virus (AMV) reverse transcriptase; *Escherichia coli* ribonuclease H (RNase H); and T7 DNA dependant RNA polymerase. During a NASBA reaction (Figure 1.4), the forward primer (P1), containing a 5'-end T7 promoter sequence, hybridises to the 3'-end of the ssRNA target. Reverse transcription extension from the 3'-end of this hybridised forward primer synthesises a complementary DNA strand, followed by RNase H activity degrading the original RNA strand from this newly formed DNA/RNA hybrid. The reverse primer (P2) hybridises to the 5'-end of the new antisense ssDNA and reverse transcription extension forms dsDNA. The T7 RNA polymerase can now target the T7 promoter site incorporated in this newly formed dsDNA, transcribing the sense DNA strand and producing antisense RNA. These RNA strands are now targeted by the reverse primer in the same manner that the original RNA template was targeted by the forward primer, repeating the above amplification process. These newly produced antisense RNA and complementary DNA templates serve as targets for continuous NASBA cycling, resulting in the exponential amplification of antisense RNA which is complementary to the original target [60].



**Figure 1.4: NASBA reaction schematic [60].**

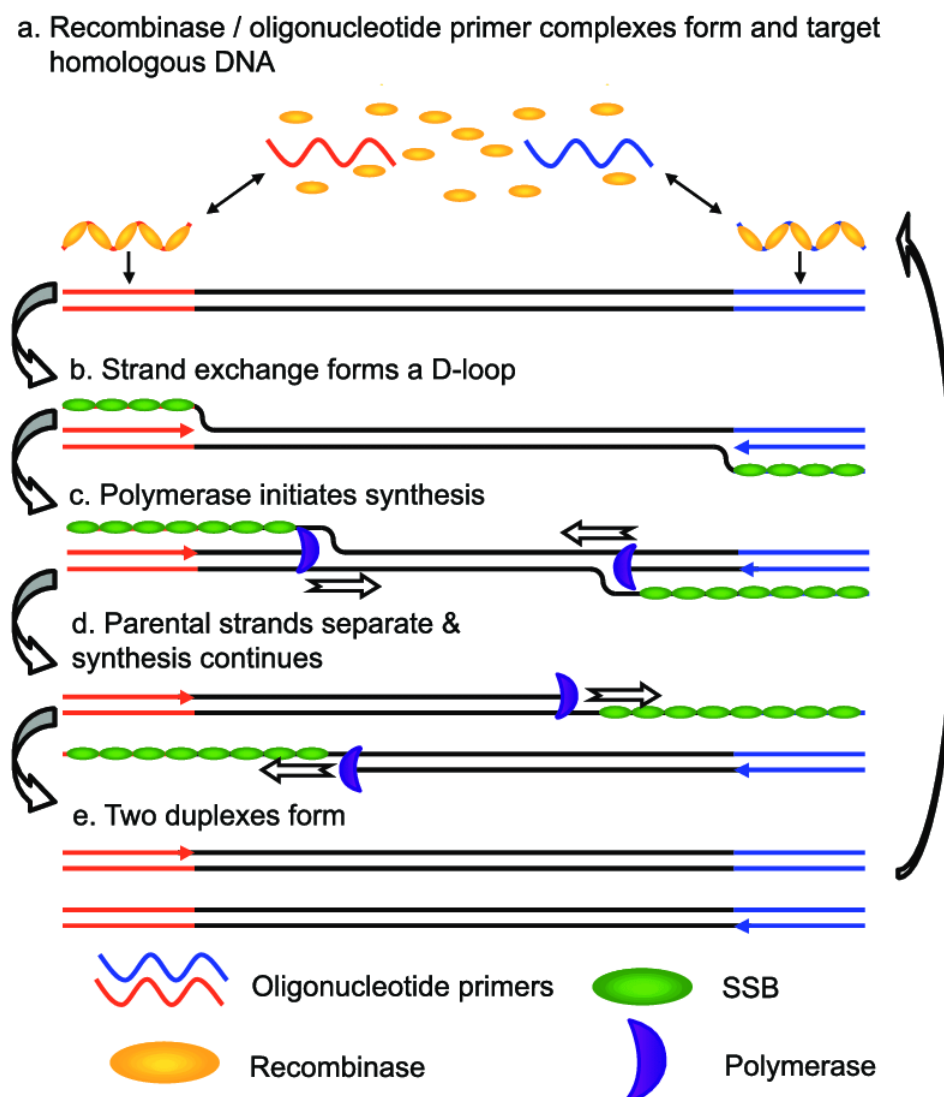
NASBA reactions are performed at a single temperature of 41°C, however, initial template denaturation at 90-95°C is required to remove RNA secondary structures before the addition of enzymes, resulting in this technology not being completely isothermal. The analytical specificity and sensitivity of NASBA is comparable to that of RT-PCR, and reactions produce high amplicon yields of approximately  $10^9$  target copy concentrations within 1-2 hours. Monitoring of NASBA reactions can be performed using post-amplification gel electrophoresis, colorimetric and electrochemiluminescence analysis. Real-time monitoring of NASBA reactions is typically performed by the incorporation of fluorescently labelled molecular beacons. NASBA has been extensively applied for the detection of various infectious disease associated pathogens [60].

### *1.3.2 Recombinase polymerase amplification (RPA)*

Recombinase polymerase amplification (RPA) is a rapid isothermal nucleic acid amplification method developed by Cambridge-based UK company, TwistDx [61]. This technology offers one of the fastest nucleic acid amplification diagnostic approaches available, providing single-digit genome copy detection within 5-10 min. A unique feature of RPA, in addition to being an isothermal technology, is that it can provide this rapid amplification at low temperature ranges of 37-42°C [62]. RPA also provides very robust target amplification in the presence of high volumes of inhibiting exogenous nucleic acids, essential when dealing with crude samples [63, 64]. The sensitivity and specificity of RPA is comparable to that of real-time PCR and it is compatible with standard end-point and real-time fluorescence monitoring methodologies. TwistDx offers various standardised RPA assay kits using different oligonucleotide and reagent conformations for different diagnostic applications: TwistAmp® basic; TwistAmp® exo; TwistAmp® exo RT; TwistAmp® fpg; and TwistAmp® nfo. All RPA kits utilise target specific forward and reverse oligonucleotide primers with three enzymes: recombinase; strand displacement DNA polymerase; and single-stranded binding proteins. During RPA reactions (Figure 1.5), the oligonucleotide primers and recombinase enzyme form a primer-protein complex that scans the dsDNA target for homologous sequence matches. After locating the specific target, the primer-protein complex unwinds the DNA forming a D-loop structure which is stabilised by the single-stranded binding proteins. DNA polymerase binds to the 3'-end of the hybridised primer-protein complex and initiates strand displacement amplification. Single-stranded DNA is produced creating additional sites for the primer-protein complexes to target and exponential amplification to occur [62].

RPA forward and reverse primers are longer than PCR primers, at 30-35 bases. This enables efficient coordination with the recombinase enzyme to form the primer-protein complex, however, previous studies have demonstrated that shorter PCR length primers can also be used [65]. TwistDx have established various RPA primer design parameters to help improve the formation of the unique primer-protein complex, such as, 30-35 base lengths, avoidance of guanine tracks with inclusion of cytosine and thymine residues near 5'-ends, and presence of guanine and cytosine residues towards 3'-ends. RPA primer design generally involves screening

combinations of 5-10 forward and reverse primer sets to find an optimal pair, using a typical amplicon length guide of 100-200 bases [66].



**Figure 1.5: RPA reaction schematic [67].**

Real-time monitoring of RPA reactions, using thermostatic fluorescence acquisition instrumentation, can be performed using standard DNA intercalating dyes or the TwistAmp® exo assay. This assay incorporates all components of the TwistAmp® basic kit with the addition of a restriction enzyme, exonuclease III, and a fluorescently labelled RPA exo oligonucleotide probe. Similar to real-time PCR hydrolysis probes, the RPA exo probe is positioned between the forward and reverse RPA primers and is longer than these primers (44-52 bases) with a higher  $T_m$  value ensuring it binds to the target first. RPA exo probes are designed with an internal cleavage site called a tetrahydrofuran (THF) spacer, which is flanked by a fluorophore and quencher

coordinated to nearby thymine residues. The THF spacer is cleaved by exonuclease III when the exo probe is in target hybridised double-stranded form, enabling fluorophore and quencher dissociation via RPA strand displacement, leading to fluorescence production. Additionally, after cleavage of the exo probe, a free 3'-end hydroxyl group is created providing an extra site for strand displacement polymerase extension to occur. The RPA exo probe THF spacer is located 15 bases from the 3'-end to ensure efficient exonuclease III binding and cleavage. There is also a typical 30 base maximum and 15 base minimum length from the THF spacer and the 5'-end of the exo probe. The fluorophore and quencher coordinated thymine residues, on either side of the THF spacer, are approximately 4-6 bases apart ensuring efficient quenching. In the TwistAmp® exo assay, a phosphorothioate 3'-end block is placed behind the last base on the probe and primers to prevent exonuclease III degradation. Additionally, a C3 Spacer is incorporated at the 3'-end of the exo probe to prevent unwanted polymerase extension which would lead to primer-dimer formation and non-specific signal generation. When designing TwistAmp® exo assays, the primers can overlap the 5'-end of the exo probe by 10-15 bases, however, this overlap cannot occur at the 3'-end as non-specific signal generation would occur. RPA technology can also be used to amplify RNA targets using the TwistAmp® exo RT kit. In combination with the basic RPA assay components, the RT kit contains reverse transcriptase, enabling single reaction real-time RNA detection [66]. The effective application of RPA as a low-cost, simple-to-use, infectious disease POC diagnostic method has been demonstrated with integration on various field-deployable, microfluidic platforms [68-72].

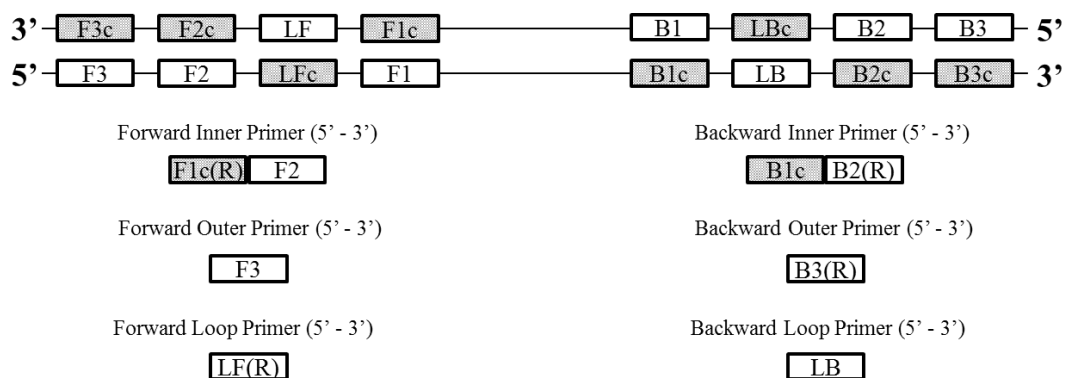
### *1.3.3 Loop-mediated isothermal amplification (LAMP)*

Loop-mediated isothermal amplification (LAMP) is specific and sensitive isothermal nucleic acid amplification technique developed by Eiken Chemical Co., Ltd, and has been extensively applied in the area infectious disease diagnostics [57]. LAMP comprises of six target specific oligonucleotide primers in combination with the strand displacement DNA polymerase enzyme, *Bacillus stearothermophilus* (*Bst*). The LAMP primers include forward and reverse sets of inner, outer and loop primers, commonly referred to as forward outer (F3) and backward outer (B3), forward inner

(FIP) and backward inner (BIP), and forward loop (LF) and backward loop (LB). The first report of LAMP by Notomi and colleagues in 2000 used only the outer and inner primer sets [73]. In 2002, Nagamine and colleagues further expanded on this technology by incorporating loop primers [74]. The combination of loop primers with the existing outer and inner sets significantly accelerated the amplification process, halving the time required for LAMP reactions. This rapid amplification results in single-digit genome copy sensitivity and high accumulation of  $10^9$  target copies in 0.5-1 hours. LAMP primers target 8 distinct regions on the target DNA making it a highly specific nucleic acid amplification method. Reactions are performed at a single temperature ranging from 60-65°C. At this temperature range the target DNA is in a state of dynamic equilibrium, enabling template dissociation and primer hybridisation. Betaine, a commonly used optional component of LAMP, further enables this template dissociation by lowering the melting temperature of the target DNA, improving primer hybridisation. Betaine is also commonly used in LAMP and other nucleic acid amplification methods to reduce secondary structure formation, improving amplification and enhancing specificity [75]. The addition of reverse transcriptase to LAMP reactions, referred to as RT-LAMP, enables the amplification of RNA templates in a single LAMP reaction without a prior reverse transcription step [76]. Novel strand displacement DNA polymerases with additional reverse transcriptase activity can also be used in LAMP reactions, negating the requirement of two separate enzymes for RNA amplification [77]. This approach enables the detection of both DNA and RNA, however, when targeting RNA prior removal of residual DNA with DNase treatment is required to prevent false positives.

The design of LAMP oligonucleotide primers is typically performed using primer design software, such as PrimerExplorer V4/V5. However, Eiken Chemical Co., Ltd have established various guidelines for manual LAMP primer design, such as, optimal primer length and spacing,  $T_m$  values, and guanine/cytosine composition parameters. LAMP assay primer design in relation to its DNA target template can be seen in Figure 1.6. The inner primers (FIP/BIP) are composed of sense and antisense strands which facilitate the formation of the unique single-stranded loop structures in LAMP. The forward inner primer (FIP) consists of the F2 sequence at its 3'-end and the reverse of F1c sequence at its 5'-end. This results in the 3'-end of the FIP primer being complementary to the F2c target sequence, and the 5'-end the FIP primer being

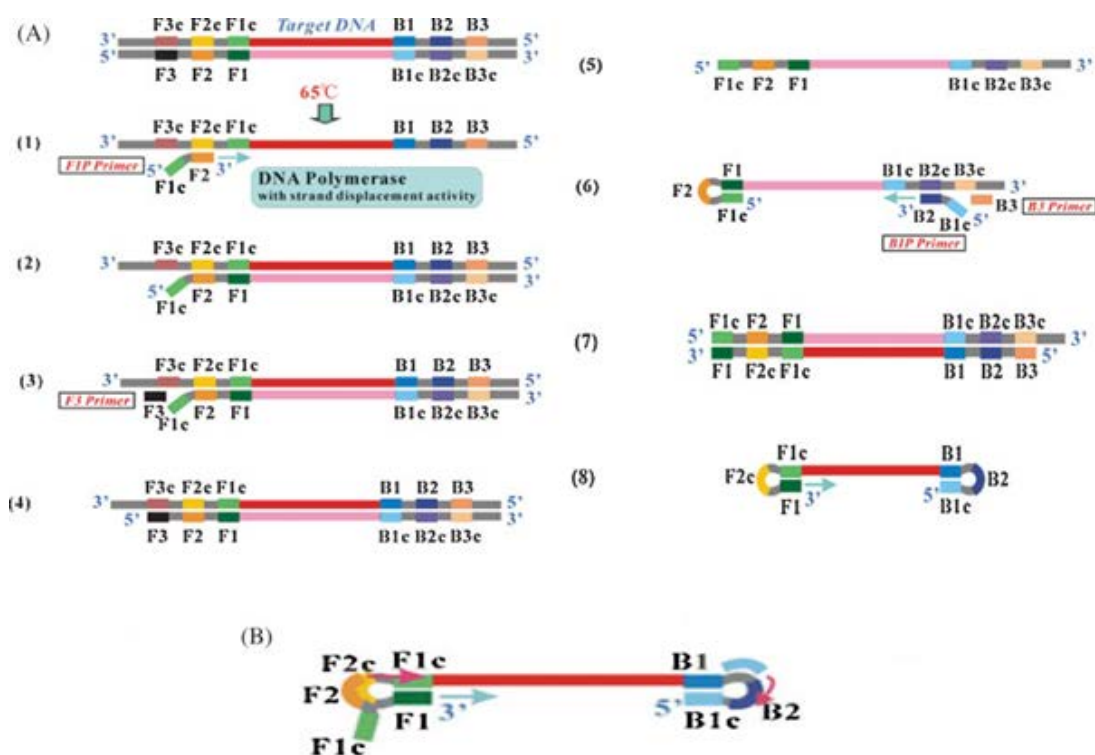
complementary to the F1 target sequence. The forward outer primer (F3) consist of the F3 sequence making it complementary to the F3c target sequence, and the forward loop primer (LF) consists of the reverse of the LF sequence, making it complementary to the LFc target sequence. The backward inner, outer and loop primers are arranged in a mirrored reverse conformation to the forward primer set [78].



**Figure 1.6: LAMP oligonucleotide primers.** Schematic representation of the outer, inner and loop primer locations in relation to the target DNA.

The inner primer sequence is formed by the separate design of its 3' sense and 5' antisense sequences. The 3' sense sequence of the inner primer is typically 16-24 bases long with a  $T_m$  value of 59-61°C. The 5' antisense sequence of the inner primer is usually 20-24 bases long with a  $T_m$  value of 64-66°C. Outer primers are 16-24 bases long with  $T_m$  values of 59-61°C, and the loop primers are also 16-24 bases long with a higher  $T_m$  value of 64-66°C. Guanine/cytosine content of LAMP primers should be between 40-65% and the adenine/thymine content between 40-50%. The 3' ends of LAMP primers should not be AT rich or complementary with the other primers so as to reduce the chance of secondary structure formation. LAMP amplicon length, the distance between the outer ends of the forward and reverse primer sets, is typically 140-180 bases long. The minimum distance between the two sets of forward and reverse primers can be as low as 0, but overlap cannot occur. Distances between the outer primers and the 3' sense sequence of the inner primers, is usually 0-20 bases long. The length of the loop section, the region between the sense and antisense sequences of the inner primer and the total sequence region that the loop primer can target, should be 40-60 bases long. Loop primers cannot overlap or be complementary to the 5' antisense sequence of the inner primer as this would inhibit loop formation, but can overlap the 3' sense sequence of the inner primer by up to 5-7 bases [78].

The mechanism of LAMP target amplification is based on auto cyclic strand displacement at a constant temperature, consisting of a non-cyclic and cyclic phase. The non-cyclic LAMP reaction phase forms a unique double looped template structure which acts as the starting point for exponential amplification in the cyclic LAMP phase. The relatively high temperature of LAMP, ranging from 60-65°C, and the optional presence of betaine, causes dissociation of the target template placing the DNA in a state of dynamic equilibrium. This dissociation enables the F2 region of the inner FIP primer to bind to its complementary F2c region [Figure 1.7 (A1)].



**Figure 1.7: LAMP mechanism.** (A) Non-cyclic amplification phase of the LAMP reaction. (B) Cyclic amplification phase of the LAMP reaction [75].

The *Bst* DNA polymerase then binds to the 3' end of this hybridised primer and polymerase extension occurs [Figure 1.7 (A2)]. The outer F3 primer binds to its complementary F3c region upstream of the hybridised inner FIP primer [Figure 1.7 (A3)] leading to strand displacement polymerase extension releasing the FIP-linked strand [Figure 1.7 (A4-5)]. The F1c sequence of this newly released FIP-linked strand hybridises to its complementary F1 region on the same strand, forming a loop structure [Figure 1.7 (A5-6)]. Upstream of this loop structure, the B2 sequence of the inner BIP primer binds to its complementary B2c region and strand displacement polymerase

extension dissociates the downstream loop structure [Figure 1.7 (A6-7)]. The outer B3 primer binds upstream of the hybridised inner BIP primer leading to strand displacement polymerase extension releasing the BIP-linked strand [Figure 1.7 (A7-8)]. This released BIP-linked strand forms loop structures at each end due to complementarity between the F1 and F1c regions, and the B1c and B1 regions [Figure 1.7 (A8)]. This unique double looped structure is the starting point for the cyclic amplification phase of LAMP which utilises the inner and loop primers. During the cyclic phase, the F2 sequence of the inner FIP primer hybridises to its complementary F2c sequence in the loop region, and the LB loop primer binds to its complementary section between the B1 and B2 regions on the opposing loop structure [Figure 1.7 (B)]. Strand displacement polymerase extension now initiates from all available 3'-end sites: the inner FIP primer; the LB loop primer; and the F1 3'-end of double looped template, leading to dissociation of both loop structures. The above amplification process is mirrored by the reverse set of LAMP primers during the reaction. These repeating phases of non-cyclic and cyclic amplification lead to the rapid exponential production of LAMP amplicon which consists of alternately inverted repeats of the target sequence [75].

Various methods can be used to examine LAMP reactions. Direct visualisation of LAMP is possible due to the high production of amplification by-product, magnesium pyrophosphate, causing reactions to become turbid or cloudy [79]. Direct visualisation is also possible with the prior incorporation of intercalating fluorescent dyes into the LAMP reaction followed by UV analysis [80]. Calcein is a chelating fluorescent reagent that is quenched when added to a LAMP reaction in the presence of manganese chloride, however, pyrophosphate LAMP by-product causes calcein to fluoresce, enabling visual amplicon detection [81]. Post-amplification visualisation of LAMP amplicons is possible using gel electrophoresis in combination with intercalating dyes and UV analysis, typically resulting in distinct ladder like structures. As previously mentioned, this post-amplification analysis method leads to contamination issues from open reaction tubes, which is especially prevalent with the high amplicon production in LAMP [82]. Real-time monitoring of LAMP reactions is possible using turbidimetric analysis of the white precipitate produced during LAMP reactions [83]. Real-time detection of LAMP reactions is most commonly performed using fluorescent intercalating dyes in combination with thermostatic fluorescent detection

equipment [84]. Alternative real-time monitoring of LAMP reactions can be achieved using colourimetric or pH sensitive dyes [85, 86].

LAMP is user-friendly, cost-effective, robust, capable of amplifying nucleic acids from samples without prior extraction, and compatible with basic POC detection technologies, making it an ideal near-patient infectious disease diagnostic option [87-89]. However, conventional monitoring methods of LAMP are not sequence specific and the amplicons produced are structurally complex making identification or multiplex differentiation complicated. Also, the non-exonuclease strand displacement activity of *Bst* DNA polymerase in LAMP is not compatible with standard nucleic acid hybridisation probes, making multiplex detection very difficult [90]. Clinical application of nucleic acid diagnostics requires multiplex detection capabilities for simultaneous pathogen detection, reduced analysis time, conservation of sample and incorporation of assay validating internal controls [51, 52].

#### **1.4 Bacterial meningitis**

Bacterial meningitis is an infectious disease caused by human commensal bacteria that invade the respiratory tract and central nervous system (CNS) resulting in inflammation of the meninges and the rapid onset of symptoms such as stiff neck, headache, fever and nausea, which if left untreated can result in death [91]. The pathophysiology of bacterial meningitis infection involves initial colonisation followed by entry into the bloodstream. Various bacterial meningitis associated pathogens colonise and penetrate the upper respiratory tract or nasopharyngeal mucous membranes by a combination of cell surface attachment, using fimbriae or pili, and avoidance of the hosts defence mechanisms. Following this, bacteria enter the bloodstream by passing directly through, or between cells, and are dispersed via the host's circulatory system. The majority of bacterial meningitis cases occur due to the presence of bacteria in the blood, however, direct dissemination to the CNS is very common. The invading bacteria will eventually access the subarachnoid space of the CNS via haematogenous spread or by direct dissemination from a particular contiguous location. The subarachnoid space is typically lacking in host immunity mechanisms which enables the rapid multiplication of the infecting bacteria. Host pattern recognition receptors identify bacterial components in the cerebrospinal fluid

leading to the induced production of inflammatory mediators. The severity of this inflammation and subsequent damage to the CNS is dependent on the unique interaction between the host and the particular pathogenic bacterium. Inflammation leads to the influx release of cytokines and granulocytes that cross the blood-brain barrier causing increased permeability and transendothelial migration of white blood cells. This increased blood-brain barrier permeability can result in plasma protein leakage causing cerebral edema, intracranial pressure and impaired circulation which can contribute to neurologic damage and eventually death. Lysis of the invading bacteria occurs when stationary growth phase is reached, leading to the additional release of inflammatory agents [92-95].

#### *1.4.1 Etiology and epidemiology of bacterial meningitis*

*Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* are the most common etiological agents of bacterial meningitis infection, as well as other invasive diseases such as pneumonia or sepsis. *S. pneumoniae* and *N. meningitidis* are responsible for over 80% of all bacterial meningitis cases since the implementation of the *H. influenzae* type b (Hib) conjugate vaccine. However, *H. influenzae* related meningitis remains a serious threat in regions without vaccination programmes and especially amongst children under five years of age [96-98]. Serogroup or serotype categorisation of these three organisms is based on polysaccharide capsule structure. Currently, 24 *S. pneumoniae* serotypes, 6 *N. meningitidis* serogroups and 6 *H. influenzae* serotypes are identified as the leading causes of infection [99]. The distribution of these particular serogroups or serotypes varies by location and demonstrates a significant annual global pattern with greater rates of incidence during winter months in both Northern and Southern hemispheres [100].

The occurrence of bacterial meningitis infection in developed regions, such as Europe and the USA, is approximately 1 in 100,000 persons per year. The incident rate of bacterial meningitis infection in developing regions, such as Africa and Southeast Asia, is approximately 20-40 in 100,000 persons per year [101]. Associated mortality of bacterial meningitis infection can be as high as 10-30% in developed countries and close to 50% in disease-burdened developing regions, even with optimal therapy. Almost half of all bacterial meningitis survivors are left with severe neurological or

motor impairment [99, 102, 103]. Bacterial meningitis infection incident rates are generally age specific with neonates under 2 months of age at most risk, followed by children between the ages of 2-24 months. These incident rates decline amongst children aged 2-10 years old and further decline amongst children at 11-17 years of age. However, rate of infection increases for adults over the age of 65 [104].

The introduction of global immunisation programmes utilising new vaccines has altered the epidemiology of bacterial meningitis infection [99]. During the early 1990s in developed countries, *H. influenzae* was responsible for approximately 45% of all cases of bacterial meningitis infection, with *S. pneumoniae* and *N. meningitidis* accounting for 18% and 14%, respectively [105]. In countries that introduced *H. influenzae* type b (Hib) immunisation programmes, cases of bacterial meningitis related infection dropped by 95-99% [101, 106]. The introduction of heptavalent and 13-valent pneumococcal vaccines in various developing countries reduced related cases of pneumococcal infection by up to 50%, with the most significant reduction in infant patients under 2 years of age or elderly patients over the age of 65 [107-109]. Meningococcal vaccines have caused a significant reduction in related cases of meningococcal infection in developing countries mainly due to herd immunity [110]. Although the implementation of global vaccination programmes has significantly reduced the occurrence of bacterial meningitis infection, approximately 1.2 million cases and over 100,000 related deaths are still reported annually, with increasing incidents of drug resistance leading to treatment failures [103, 111].

#### 1.4.2 Bacterial meningitis diagnosis

The rapid and accurate detection of bacterial meningitis pathogens is essential for early and correct antibiotic administration, which is directly linked with lowering associated morbidity and mortality. Additionally, accurate diagnosis prevents over-prescription of broad-range antibiotics, a major contributing factor to the increasing threat of antimicrobial-resistance dissemination [112, 113]. The initial diagnosis of bacterial meningitis infection based on direct examination of physical symptoms depends on a patient's age, with atypical clinical manifestations occurring in very young, immunocompromised or elderly patients. The classic indicators of meningitis infection in both children and adults are fever, headache, neck stiffness, vomiting,

photophobia and seizures [106, 114]. Infants experience non-specific symptoms such as hypothermia, lethargy, high-pitched crying, no appetite, apnea or cranial swelling. Older children can experience increased seizures when infected with *S. pneumoniae* and *H. influenzae* compared to *N. meningitis*. Common signs of infection in patients over the age of 65 can be altered mental state and confusion [114, 115]. Direct diagnosis of bacterial meningitis infection can be difficult due to the presentation of non-differentiable symptoms, such as neck stiffness, headache and fever, occurring in most cases [116], requiring laboratory-based testing for confirmation of infection. Conventional laboratory-based diagnosis of bacterial meningitis infection involves Gram-staining and bacterial culture examination of blood or cerebrospinal fluid (CSF) samples. These methods enable identification of causative pathogens for determination of correct antibiotic treatment and susceptibility testing. CSF analysis is the gold standard for the diagnosis of bacterial meningitis infection, with elevated CSF pressure being a classic indicator of infection [116], as well as high white blood cell counts [99]. The CSF samples from infected patients are generally low in glucose compared to serum values and contain relatively high protein levels. Measuring CSF lactate provides very sensitive and specific indication of infection with effective differentiation between bacterial and viral agents, especially if analysed prior to antibiotic treatment [117]. Analysis of CSF procalcitonin levels is also used to identify meningitis caused by a bacterial pathogen [118]. Timely analysis of CSF samples is essential if lumbar puncture is delayed until after antimicrobial treatment, with successful pathogen identification in these cases being reduced by over 40% [119].

Various molecular diagnostic technologies offer alternative approaches to conventional culture-based methods for the diagnosis of bacterial meningitis infection, with real-time PCR being the most commonly applied technology. Real-time PCR typically utilises the 16S rRNA universal biomarker gene to successfully detect causative bacterial meningitis pathogens in CSF and blood samples with high sensitivity and specificity, even in cases of prior antibiotic treatment [120-123]. Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry is a high-throughput protein sequencing technology used to identify organisms. MALDI-TOF is routinely used for the detection of bacterial meningitis pathogens in CSF samples [124]. Fluorescent in-situ hybridization (FISH) has been applied to the identification of bacterial meningitis associated pathogens. This method utilises

fluorescently labelled oligonucleotide probes that rapidly target nucleic acid sequences of microorganism enabling visualisation without the need for prior culturing [125-128]. Whole genome sequencing analysis methods such as pyrosequencing, dye sequencing and nanopore sequencing have also been effectively applied to the identification of bacterial meningitis pathogens [129, 130].

However, these current methodologies for the diagnosis of bacterial meningitis infection can have limitations in terms of speed, specificity, sensitivity and application in highly disease-burdened areas with poor infrastructure and low resources. Blood and CSF culture is a time consuming method that can often lead to false negatives in the case of viable non-culturable organisms, with poor reported sensitivities in some cases [128, 131-133]. As previously discussed, real-time PCR is an impractical POC diagnostic option for bacterial meningitis infection in low-resourced areas due to the requirement of expensive thermocycling equipment. MALDI-TOF requires a culture step prior to analysis which is limiting in cases of fastidious bacterial meningitis pathogens or in cases of prior antimicrobial treatment reducing culture sensitivity [134]. Also, the identification of bacterial meningitis associated organisms without reference spectra on MALDI-TOF mass spectroscopy databases is difficult [127, 135]. Various studies using FISH for the identification of bacterial meningitis associated pathogens have highlighted shortcoming in terms of specificity and false positives [127, 128]. Whole genome sequencing is a reliable detection method, however, the requirement of specialised equipment, skilled operation and time needed to perform this approach makes it an impractical option for low-resourced areas, especially in terms of near-patient POC application [89]. Furthermore, most of these bacterial meningitis diagnostic methods do not enable rapid serogroup or serotype identification for effective disease surveillance and appropriate public health care containment response.

## **1.5 Research aims**

The primary focus of this thesis was to advance the current state-of-the-art in multiplex isothermal nucleic acid amplification detection of the major bacterial meningitis pathogens, *S. pneumoniae*, *N. meningitidis* and *H. influenzae*, for potential use in near-patient POC testing. This project was conducted with the Molecular Diagnostics

Research Group (MDRG), at the National University of Ireland, Galway, led by Prof. Terry Smith, whose research team have extensive experience in the development of infectious disease diagnostics. To achieve the research aims of this project, focus was placed on the design, development and evaluation of two isothermal nucleic acid diagnostic technologies, recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP). RPA, a uniquely rapid isothermal diagnostic method developed by the Cambridge-based UK company, TwistDx, utilises novel oligonucleotide probe technology to monitor reactions in real-time. We aimed to gain essential experience in the design and development of RPA assays for the detection of all three bacterial meningitis pathogens. This knowledge gained would subsequently be applied to the design and development of novel real-time probing methods for LAMP technologies, also targeting these three pathogens. Various domestic and international diagnostic-based manufactures, such as HiberGene and DiaSorin, have successfully launched commercial LAMP-based tests, however, this requires expensive licensing from LAMP developers Eiken Chemical Co., Ltd. The current LAMP technology patent is expected to expire in 2018/2019 presenting possible commercialisation opportunities around novel applications of this technology, which this project hopes to explore [136].

Design and development of the nucleic acid amplification technologies in this project began with the establishment of diagnostic targets for *S. pneumoniae*, *N. meningitidis* and *H. influenzae*. The RPA and LAMP technologies detailed in Chapters 2, 3 and 4 of this thesis utilised a novel *N. meningitidis* target, the *NMO\_1242* gene, which was identified in this project using whole genome sequence alignment analysis of publically available sequences. The RPA assays in Chapter 2 of this thesis used the established biomarkers elongation factor 4 (EF4)/*lepA* gene and L-fucose kinase/*fucK* gene, for the detection of *S. pneumoniae* and *H. influenzae*, respectively. The *S. pneumoniae* and *H. influenzae* targets used in the LAMP technologies detailed in Chapters 3 and 4 of this thesis were the *SPNA45\_01710* and *pstA* genes, respectively. Recently published research, linked with the MDRG, established the *pstA* gene as an effective diagnostic target for *H. influenzae*, and the *SPNA45\_01710* gene was identified in this project as an effect biomarker using whole genome alignment sequence analysis. RPA and LAMP assay development involved the initial design of singleplex assays for the detection of each organism, followed by

development of multiplex assays for the simultaneous detection of all organisms. Assays were optimised in terms of oligonucleotide/reagent composition and reaction parameters such as temperature, and evaluated in terms of analytical specificity and sensitivity using inclusivity and exclusivity panels of bacterial reference strains. Small scale clinical sample testing for each assay assessed clinical performance using archived genomic DNA from confirmed cases of pneumococcal, meningococcal and Haemophilus infection. Development of the RPA technologies in this project was a collaborative effort between the MDRG and TwistDx, with the Galway University Hospital and the Irish Meningitis and Sepsis Reference Laboratory (IMSRL), Dublin, supplying all clinical isolates and samples tested.

Chapter 2 of this thesis describes the development and evaluation of three duplex RPA assays, incorporating competitive internal amplification controls, for the detection of *S. pneumoniae*, *N. meningitidis* and *H. influenzae*. Singleplex RPA assays were initially designed and evaluated demonstrating 100% specificity with single-digit genome copy limit of detection (LOD). This work built on previously published research that was conducted as part of this thesis detailing the development of a singleplex RPA assay for the detection of *S. pneumoniae* in whole blood [137]. Internal amplification control templates and corresponding RPA oligonucleotides were incorporated into each singleplex assay creating internally controlled duplex RPA assays. The clinical performance of each duplex RPA assay was assessed using DNA extracts from confirmed cases of bacterial meningitis infection, supplied by the IMSRL. Compared to PCR, the RPA assays demonstrated 100% diagnostic specificity, with diagnostic sensitivities of 100%, 85.7% and 100% for the *S. pneumoniae*, *N. meningitidis* and *H. influenzae* assays, respectively. This study detailed the first report of internally controlled duplex RPA assays for the detection of these major bacterial meningitis pathogens.

In Chapter 3 of this thesis we introduced *Tth* endonuclease cleavage loop-mediated isothermal amplification (TEC-LAMP) technology, a novel real-time multiplex LAMP assay for the internally controlled detection of *S. pneumoniae*, *N. meningitidis* and *H. influenzae*. This technology was developed through the use of a novel TEC primer/probe and the restriction enzyme *Tth* endonuclease IV. The analytical specificity, LOD and clinical application of the TEC-LAMP assay was evaluated. TEC-LAMP was 100% specific, with LODs for *S. pneumoniae*, *N. meningitidis* and

*H. influenzae* of 39.5, 17.3 and 25.9 genome copies per reaction, respectively. The diagnostic sensitivity and specificity of the TEC-LAMP assay was established to be 92.3% and 100%, respectively, when tested with a range of PCR-positive DNA extracts from cases of bacterial meningitis infection. This study detailed the first report of a single-tube internally controlled multiplex LAMP assay for the detection of bacterial meningitis pathogens, and the first report of *Tth* endonuclease IV incorporation into nucleic acid amplification diagnostic technology.

Chapter 4 of this thesis detailed a significant advance on the previous TEC-LAMP technology, introducing loop-primer endonuclease cleavage loop-mediated isothermal amplification (LEC-LAMP). LEC-LAMP also enables real-time multiplex bacterial meningitis pathogen detection, however, this technology incorporates flexible single-base specificity for effective single nucleotide polymorphism (SNP) detection. LEC-LAMP utilises a novel LEC primer/probe with the restriction enzyme endonuclease IV. Development of this technology began with the design of a singleplex LEC-LAMP *N. meningitidis* assay. Evaluation of this assay demonstrated complete analytical specificity, a LOD of 3.1 genome copies per reaction and 100% diagnostic specificity and sensitivity when assessed with anonymised DNA extracts from confirmed cases of bacterial meningitis infection. Additionally, this assay demonstrated effective single-base specificity when challenged with DNA templates containing single nucleotide mismatches anywhere in a specific 6 base target region. Modifications to this assay successfully demonstrated single-tube wild-type and mutant allele differentiation, as well as simultaneous multiplex target detection of *N. meningitidis*, *S. pneumoniae* and *H. influenzae*. LEC-LAMP is the first report of a single-tube real-time multiplex LAMP technology with flexible single-base specificity for SNP identification.

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## **Chapter 2**

### **Duplex Recombinase Polymerase Amplification Assays Incorporating Competitive Internal Controls for Bacterial Meningitis Detection**

#### **Published by Analytical Biochemistry**

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#### **Author Contributions**

OH, EC, MSF, OP and TJS designed the study. OH carried out all experimental work and drafted the manuscript. MC, TWB, NOS, CMG, DC and RC were involved in the collection and analysis of clinical samples. All authors contributed to the review of the final manuscript.

## Abstract

Recombinase polymerase amplification (RPA) is an isothermal nucleic acid amplification technology that provides rapid and robust infectious disease pathogen detection, ideal for point-of-care (POC) diagnostics in disease-prevalent low-resource countries. We have developed and evaluated three duplex RPA assays incorporating competitive internal controls for the detection of predominant bacterial meningitis pathogens. *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* singleplex RPA assays were initially developed and evaluated, demonstrating 100% specificity with limits of detection of 4.1, 8.5 and 3.9 genome copies per reaction, respectively. Each assay was further developed into internally controlled duplex RPA assays via the incorporation of internal amplification control templates. Clinical performance of each internally controlled duplex RPA assay was evaluated by testing 64 archived PCR-positive clinical samples. Compared to real-time PCR, all duplex RPA assays demonstrated 100% diagnostic specificity, with diagnostic sensitivities of 100%, 85.7% and 100% for the *S. pneumoniae*, *N. meningitidis* and *H. influenzae* assays, respectively. This study details the first report of internally controlled duplex RPA assays for the detection of bacterial meningitis pathogens: *S. pneumoniae*, *N. meningitidis* and *H. influenzae*. We have successfully demonstrated the clinical diagnostic utility of each duplex RPA assay, introducing effective diagnostic technology for POC bacterial meningitis identification in disease-prevalent developing countries.

## 2.1 Introduction

Bacterial meningitis is a life-threatening infectious disease, typically caused by invasive human commensal bacteria. Infection is associated with acute onset of clinical features such as headache, photophobia, fever, nausea, and neck stiffness. If bacterial meningitis infection is not appropriately treated it can lead to stroke, paralysis and death [91]. Even with appropriate treatment, mortality rates can be as high as 10-30% in developed countries and up to 50% in low-resource developing regions [99]. Post-treatment onset of bacterial meningitis sequelae, such as severe neurological or motor impairment, occurs in almost half of all survivors [103].

The leading causative agents of bacterial meningitis infection are *Streptococcus pneumoniae* and *Neisseria meningitidis*. *Haemophilus influenzae* also remains a serious threat despite introduction of the type b (Hib) conjugate vaccine, particularly amongst children under the age of 5 in areas without routine immunisation [97, 98, 100]. Advances in treatment and implementation of global vaccination programmes has significantly reduced the occurrence of bacterial meningitis infection [111]. However, approximately 1.2 million cases and over 100,000 deaths are reported annually, with increasing incidents of drug resistance leading to treatment failures [103, 111]. Also, since the implementation of vaccination programmes, resulting bacterial serotype or serogroup replacement has led to the increasing emergence of non-vaccine or non-typeable associated bacterial meningitis incidence [99].

Rapid and accurate diagnosis of bacterial meningitis infection can lower associated disease burden and prevent antimicrobial-resistance dissemination by supporting targeted antimicrobial treatment [112, 138]. Conventional laboratory diagnosis of bacterial meningitis infection involves microscopy and culture methods [99]. Identification of the causative pathogen by microscopy of cerebrospinal fluid has poor diagnostic sensitivity and does not permit definitive identification. Culture methods are time-consuming and unreliable if antimicrobial agents have been administered before samples are collected [113].

Nucleic acid amplification diagnostics offer a rapid, accurate, high-throughput, closed reaction system, capable of detecting non-viable pathogens, and are rapidly becoming the diagnostic method of choice over conventional methods in clinical settings [99, 139, 140]. Additionally, multiplex nucleic acid diagnostics enable simultaneous multiple pathogen detection, reducing analysis time and cost, and enabling incorporation of assay validating internal controls [51]. Real-time polymerase chain reaction (PCR) is the current “gold standard” nucleic acid diagnostic method, with improved clinical sensitivity (87–100%) and specificity (98–100%) over conventional culture methods [99]. However, real-time PCR requires expensive thermo-cycling equipment and skilled operation, limiting its application and further preventing its use as an effective point-of-care (POC) diagnostic technology for disease-prevalent low-resource areas.

Isothermal nucleic acid amplification methods such as loop-mediated isothermal amplification (LAMP), or helicase dependant amplification (HDA), do not require thermocycling and thus offer a simplified lower-cost alternative to real-time PCR [141]. In particular, recombinase polymerase amplification (RPA), a novel isothermal nucleic acid amplification method developed by TwistDx (Cambridge, UK), provides rapid single-digit genome copy detection within 5-10 min and robust tolerance of inhibiting exogenous nucleic acids [61, 137]. Moreover, the utility of RPA as a low-cost, simple-to-use, effective POC diagnostic tool has been demonstrated with field-deployable microfluidic platforms, making RPA an ideal diagnostics technology for highly disease-burdened developing regions [69-71].

RPA utilises target specific forward and reverse oligonucleotide primers in combination with four core proteins: recombinase; strand-displacement DNA polymerase; single-stranded binding proteins; and exonuclease III. Primer/recombinase complexes rapidly scan target DNA for sequence matches, forming D-loop structures after hybridisation which are stabilised by single-stranded binding proteins. Target-bound primer/recombinase complexes enable DNA polymerase to initiate strand-displacement amplification, leading to ssDNA production facilitating cyclical and rapid target amplification. RPA real-time monitoring is achieved using a novel exo oligonucleotide probe that incorporates a tetrahydrofuran (THF) spacer flanked by a thymine-linked fluorophore and quencher. The probe is cleaved by exonuclease III upon target hybridisation, separating the fluorophore and quencher, leading to fluorescence signal production and target detection [66].

In this study, we have successfully developed three duplex TwistAmp® exo RPA assays for the detection of *S. pneumoniae*, *N. meningitidis* or *H. influenzae*, with the simultaneous co-amplification of an internal amplification control (IAC). These RPA assays were subsequently evaluated in terms of clinical diagnostic sensitivity and specificity.

## 2.2 Materials and methods

### 2.2.1 Bacterial strains and genomic DNA preparation

A range of bacterial reference strains and clinical isolates were used to evaluate the singleplex RPA assays in this study (see Supplemental Tables 2.1, 2.2 and 2.3). The clinical isolates, supplied by Galway University Hospital (GUH), were collected between 2010 and 2015 from culture-confirmed cases of bloodstream infection as part of routine clinical diagnostic service. All bacterial strains, stored at -80°C, were cultured in brain heart infusion (BHI) media (Oxoid, Hampshire, UK) and incubated at 37°C for 18 h, under microaerophilic conditions, excluding *Haemophilus* strains which were cultured using *Haemophilus* test media (Oxoid, Hampshire, UK). The DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) was used to extract genomic DNA, and the Qubit dsDNA BR/HR assay kits with the Qubit 2.0 fluorometer (Life Technologies, Warrington, UK) were used to quantify the extracted DNA, according to manufacturer's instructions. Genome size standards of 2.1 Mb, 2.2 Mb and 1.83 Mb for *S. pneumoniae*, *N. meningitidis* and *H. influenzae*, respectively, were used to convert resulting DNA concentrations to genome copy values.

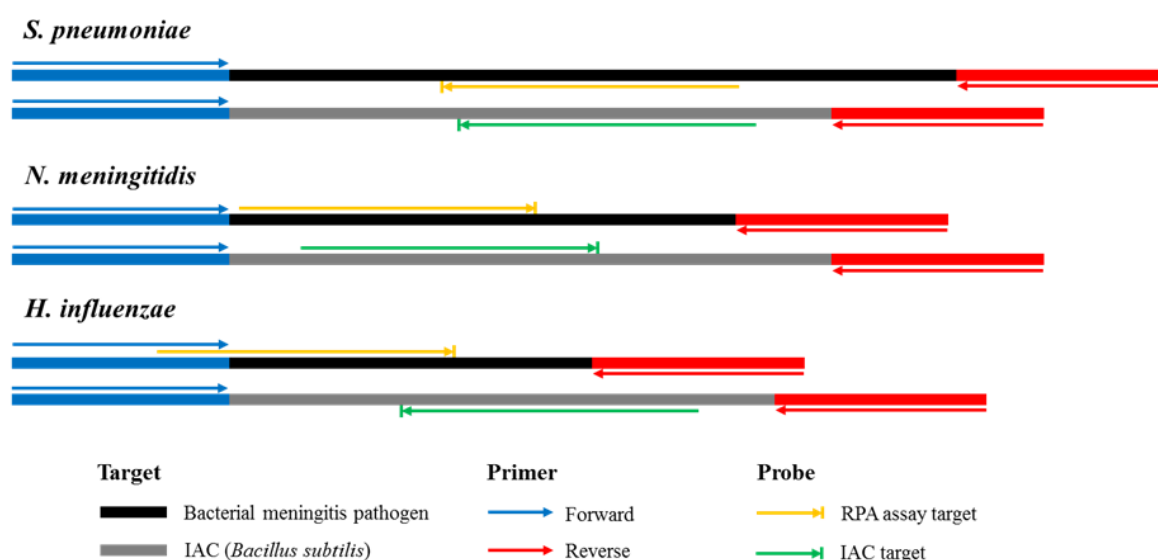
### 2.2.2 Diagnostic targets, RPA oligonucleotides and internal amplification controls

TwistAmp® exo RPA assay oligonucleotide primers and probes (Table 2.1 and Supplemental Table 2.4) were empirically designed for each bacterial target according to TwistDx guidelines (Figure 2.1). The *S. pneumoniae* assay targeted the elongation factor 4 (EF4)/*lepA* gene (position 974524-976347 of accession number HE983624.1). The *N. meningitidis* assay targeted a recently identified biomarker (Higgins, pers. comm.), *NMO\_1242*, a chromosomal cytolysin secretion ABC transporter gene (position 1295659-1297887 of accession number AM889136.1). The *H. influenzae* assay targeted the L-fucose kinase/*fucK* gene (position 643681-645517 of accession number L42023.1). A TwistAmp® exo RPA probe was also designed (Table 2.1) to target internal amplification control (IAC) templates in the *S. pneumoniae*, *N. meningitidis* and *H. influenzae* duplex RPA assays (see Supplemental Table 2.5). These IAC templates were *Bacillus subtilis* DNA PCR-products, supplied by TwistDx, consisting of the exo probe target sequence flanked by assay specific primer target sequences. All oligonucleotide primers were HPLC purified and synthesised by

Integrated DNA Technologies (Leuven, Belgium). The bacterial meningitis pathogen probes and IAC probe, labelled with FAM and TAMRA fluorophores respectively, were HPLC purified and synthesised by Biosearch Technologies (Petaluma, CA, USA). These fluorophores corresponded to one of two detection channels in the Twista® fluorometer (TwistDx), used to perform all RPA reactions.

Table 2.1: RPA oligonucleotides.	
Type	Sequence (5' - 3')
<i>S. pneumoniae</i>	
Forward Primer	ACAGCTCCGTCTGTTATTTACAAAGTTAATTTGA*C <sup>a</sup>
Reverse Primer	AGTCCCCACGCTTACGCTGAGCTAGCTCCATTAC*T
Exo Probe	CTTGACATAAGGCTCTTCAATGGTCGCAATCT[T(FAM)]A(dSpacer)[T(BHQ1)]TGGGTCTGGAAAC*T(C3 Spacer) <sup>b</sup>
<i>N. meningitidis</i>	
Forward Primer	CGTCTGTGCTCGAAATAGGATAAAGGCAGGGAAA*G
Reverse Primer	TGGCTTTGTTGGTGGTGTGCTGTTTGAGATTGT*G
Exo Probe	GCCGGAACAAACGCGCGCCAATCCACA[T(FAM)](dSpacer)AA[T(BHQ1)]ACGTGAAGTC*G(C3 Spacer)
<i>H. influenzae</i>	
Forward Primer	TTCACWACAGATCACACAATGGCGGGAACATCAA*T
Reverse Primer	CACCTGCATAACGCATAGGAGGGAAATGGTTATT*A
Exo Probe	ACATCAATGATGACAAACCTTACTAGCGG[T(FAM)](dSpacer)A[T(BHQ1)]TGGGATCCATC*G(C3 Spacer)
<b>Internal Amplification Control</b>	
Exo Probe	CGATCATGCCATCAGCAGCTTATGATCAA[T(TAMRA)](dSpacer)[T(BHQ2)]GATCCAAACCGAGGC*G(C3 Spacer) <sup>c</sup>

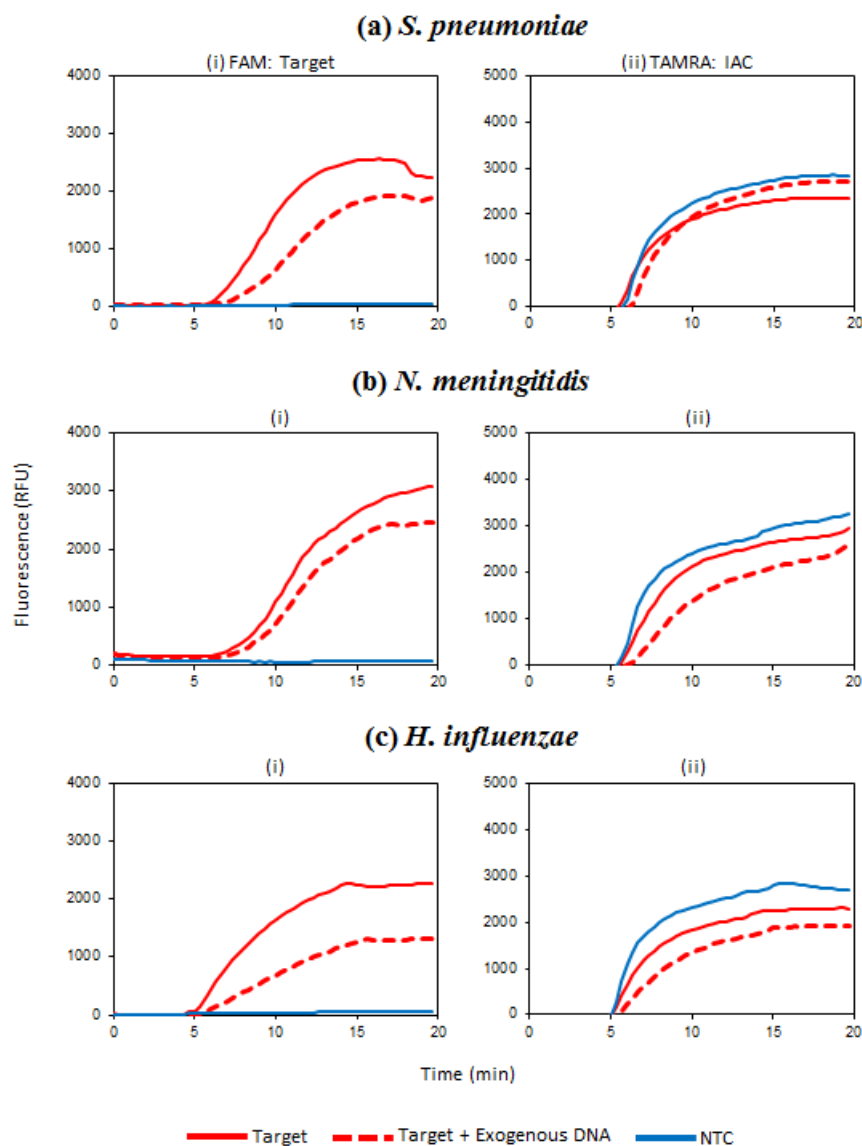
<sup>a</sup>\* = phosphorothioate bond.  
<sup>b</sup>T(FAM) = 6-carboxyfluorescein fluorophore linked to thymine; dSpacer = 1',2'-dideoxyribose; BHQ1-dT = black hole quencher 1 linked to thymine; C3 Spacer = C3 spacer phosphoramidite.  
<sup>c</sup>T(TAMRA) = tetramethylrhodamine fluorophore linked to thymine; BHQ2-dT = black hole quencher 2 linked to thymine.



**Figure 2.1: Schematic representation of the oligonucleotide primer and probe locations for each duplex RPA assay.**

### 2.2.3 Singleplex RPA assay analytical specificity and limit of detection

All singleplex RPA reactions were performed in 50  $\mu\text{L}$  volumes using TwistAmp® exo kits. Additional reaction components included 29.5  $\mu\text{L}$  primer-free resuspension buffer (PFRB), 2.5  $\mu\text{L}$  of 280 mM magnesium acetate (MgOAc), 4  $\mu\text{L}$  of 6  $\mu\text{M}$  forward and reverse RPA primers (0.48  $\mu\text{M}$  final concentration each), 1  $\mu\text{L}$  of 6  $\mu\text{M}$  TwistAmp® exo probe (0.12  $\mu\text{M}$  final concentration), 8  $\mu\text{L}$  molecular grade water, and 1  $\mu\text{L}$  DNA template or 1  $\mu\text{L}$  molecular grade water for no template control (NTC) reactions. All reagents were provided by TwistDx. The 8-tube TwistAmp® exo kit reaction strips, with the MgOAc added to the caps and the remaining reagents added to the tubes, were sealed and briefly centrifuged, vortexed and centrifuged. RPA reactions were performed at 40°C for 20 min using the Twista® fluorometer, with a brief vortex and centrifuge step after 4 min. Resulting RPA fluorescence was recorded in the FAM and TAMRA Twista® detection channels, with positive results recorded as exponential signal acquisition exceeding background fluorescence, represented as fluorescence amplification curves (Figure 2.2). Purified genomic DNA from a panel of bacterial strains (see Supplemental Tables 2.1, 2.2 and 2.3) was tested at  $5 \times 10^4$  genome copies to determine the analytical specificity of the *S. pneumoniae*, *N. meningitidis* and *H. influenzae* singleplex RPA assays. Positive control reactions testing 100 genome copies of respective type strains for each assay (*S. pneumoniae* DSM 20566, *N. meningitidis* NCTC 10025 and *H. influenzae* DSM 4690), and NTC reactions, were carried out in parallel with the analytical specificity testing. Probit analysis (Minitab) was used to establish the limit of detection (LOD) with 95% probability for each singleplex RPA assay. Respective type strains for each assay were tested using 12 replicates of 8, 7, 6, 5, 4, 3, 2 and 1 genome copy concentrations, with Probit regression analysis performed on the resulting data using Minitab 17 (see Supplemental Table 2.6).



**Figure 2.2: Demonstration of *S. pneumoniae*, *N. meningitidis* and *H. influenzae* internally controlled duplex RPA assays.** Each of the three internally controlled duplex RPA assays were challenged with 100 copies of respective bacterial targets, both in the absence (solid red) and in the presence (dashed red) of 400 ng exogenous DNA for assay robustness testing. Separate NTC reactions for each assay were performed in parallel (blue). Resulting RPA fluorescence from bacterial target amplification was recorded in the FAM detection channels of the Twistax® fluorometer (TwistDx), with IAC amplification recorded in the TAMRA detection channel. Representative amplification curves for each reaction are shown. Successful simultaneous detection of bacterial target and the IAC template ( $2 \times 10^3$  copies), both in the absence (solid red) and in the presence (dashed red) of 400 ng exogenous DNA, was observed. Assay inhibition was observed in reactions containing the exogenous DNA. The NTC reactions performed successfully as no signal was observed in the FAM channel (blue). Conversely, the NTC reactions acted as successful positive controls in the TAMRA channels (blue), as the IAC template was detected in the absence of a bacterial target.

#### 2.2.4 Development of internally controlled duplex RPA assays

The *S. pneumoniae*, *N. meningitidis* and *H. influenzae* singleplex RPA assays were further developed to establish internally controlled duplex RPA assays by incorporating an IAC template, a *Bacillus subtilis* DNA PCR-product (see Supplemental Table 2.5), and a corresponding TwistAmp® exo probe (Table 2.1). All internally controlled duplex RPA reactions were performed in 50 µL volumes using TwistAmp® exo kits modified to contain: forward and reverse RPA primers (0.48 µM final concentration each); a bacterial target exo probe (0.12 µM final concentration); an IAC exo probe (0.12 µM final concentration); and an IAC template ( $2 \times 10^3$ ,  $3 \times 10^3$ ,  $4 \times 10^3$  or  $5 \times 10^3$  copies). Additional reaction components included 37.5 µL primer-in resuspension buffer (PIRB), 4 µL of 280 mM MgOAc, 7.5 µL molecular grade water, 1 µL DNA template or 1 µL molecular grade water for NTC reactions. Reactions were performed as previously described singleplex RPA reactions. The optimum concentration of the IAC template was evaluated by testing 100 genome copies of respective bacterial targets in the presence of various IAC copy concentrations,  $2 \times 10^3$  (Figure 2.2),  $3 \times 10^3$ ,  $4 \times 10^3$  and  $5 \times 10^3$  (data not shown). Assay robustness of each optimised internally controlled duplex RPA assay was evaluated by determining the efficiency of detecting 100 genome copies of respective bacterial targets in the presence of 400 ng exogenous DNA (Figure 2.2). NTC reactions were performed in parallel as previously described.

#### 2.2.5 Clinical evaluation of internally controlled duplex RPA assays

Archived genomic DNA samples from PCR-confirmed cases of pneumococcal, meningococcal and Haemophilus infection, were used to assess the clinical performance of the *S. pneumoniae*, *N. meningitidis* and *H. influenzae* internally controlled duplex RPA assays. The Irish Meningitis and Sepsis Reference Laboratory (IMSRL), Dublin, Ireland, provided all samples, which had previously been collected as part of routine clinical diagnostic service from 2012 to 2015. Samples were anonymised residual material from routine clinical specimens. In total, 64 samples including 33 blood, 5 blood culture, 17 cerebrospinal fluid (CSF), 5 pleural fluid, 1 knee fluid and 3 other body fluids, were supplied with corresponding real-time PCR Ct-values from previous confirmatory testing carried out by the IMSRL (see

Supplemental Table 2.7). The IMSRL PCR evaluation, using standard cycling and reaction parameters, tested 2.5  $\mu$ L of each sample targeting the *S. pneumoniae* *lytA*, *N. meningitidis* *ctrA* and *H. influenzae* *fucK* genes (see Supplemental Table 2.8). For comparative purposes, the diagnostic sensitivity and specificity of the *S. pneumoniae*, *N. meningitidis* and *H. influenzae* internally controlled duplex RPA assays was also assessed by testing 2.5  $\mu$ L of each sample (see Supplemental Table 2.7). All 64 samples were tested in all three duplex RPA assays, providing negative samples for each assay. Clinical samples that were not detected by the internally controlled duplex RPA assays were re-tested using “in-house” singleplex real-time PCR assays targeting the *S. pneumoniae* *lepA*, *N. meningitidis* *NMO\_1242* and *H. influenzae* *pstA* genes (see Supplemental Table 2.9). Positive control and NTC reactions were performed in parallel as previously described.

## 2.3 Results

### 2.3.1 Singleplex RPA assay analytical specificity and limit of detection

The *S. pneumoniae*, *N. meningitidis* and *H. influenzae* singleplex RPA assays demonstrated 100% analytical specificity as all inclusivity panel strains were successfully detected, with no detection of any exclusivity panel strains observed (see Supplemental Tables 2.1, 2.2 and 2.3). All positive control reactions were successfully detected, with no signal detected in any NTC reactions. The LOD of each singleplex RPA assay, for the separate detection of *S. pneumoniae*, *N. meningitidis* and *H. influenzae*, was confirmed with 95% probability using Probit analysis to be 4.1, 8.5 and 3.9 genome copies per reaction, respectively (see Supplemental Table 2.6).

### 2.3.2 Development of internally controlled duplex RPA assays

The optimum concentration of the IAC template was determined to be  $2 \times 10^3$  copies for each internally controlled duplex RPA assay (Figure 2.2). The robustness of each optimised internally controlled duplex RPA assay was demonstrated with the successful detection of 100 copies of respective bacterial targets in the presence of 400 ng exogenous DNA (Figure 2.2). All NTC reactions performed successfully as no signal was observed in any reaction.

### 2.3.3 Clinical evaluation of internally controlled duplex RPA assays

The 64 IMSRL PCR-positive clinical samples were tested by all three *S. pneumoniae*, *N. meningitidis* and *H. influenzae* internally controlled duplex RPA assays, providing both positive and negative clinical samples for each assay (see Supplemental Table 2.7). The *S. pneumoniae* assay detected 23/23 *S. pneumoniae* samples, 0/21 *N. meningitidis* and 0/20 *H. influenzae* samples, giving a diagnostic sensitivity and specificity of 100%, when compared to the IMSRL PCR results. The *N. meningitidis* assay detected 18/21 *N. meningitidis* samples, 0/23 *S. pneumoniae* and 0/20 *H. influenzae* samples, giving a diagnostic sensitivity and specificity of 85.7% and 100%, respectively, when compared to the IMSRL PCR results. The *H. influenzae* assay detected 20/20 *H. influenzae* samples, 0/23 *S. pneumoniae* and 0/21 *N. meningitidis* samples, giving a diagnostic sensitivity and specificity of 100%, when compared to the IMSRL PCR results. The 3 *N. meningitidis* IMSRL PCR-positive samples not detected by the *N. meningitidis* assay, 24 (Ct 36.93), 40 (Ct 38.67) and 42 (Ct 36.03), were subsequently re-confirmed as positive using an “in-house” real-time PCR assay, producing similar Ct-values to the IMSRL evaluation of 36.92, 37.01 and 36.03, respectively. The IAC performed successfully during clinical sample testing as it was detected in all events of no bacterial target amplification (see Supplemental Table 2.7). All NTC reactions performed successfully as no signal was observed in any reaction.

## 2.4 Discussion

Nucleic acid amplification diagnostics offer a more rapid, sensitive and specific alternative to conventional methods for the diagnosis of bacterial meningitis; a global infectious disease with high morbidity and mortality rates, especially in developing countries [91, 99, 139, 140]. Furthermore, isothermal nucleic acid amplification diagnostics provide a simplified, lower-cost approach over “gold standard” nucleic acid technologies, such as real-time PCR [141]. In particular, RPA, a recently developed isothermal nucleic acid amplification method, offers very rapid and robust detection of infectious disease pathogens, making it an ideal POC diagnostic option for low-resource disease-burdened areas [61].

This study has evaluated the analytical specificity and LOD of three singleplex RPA assays for the detection of leading bacterial meningitis pathogens: *S. pneumoniae*, *N.*

*meningitidis* and *H. influenzae*. The 100% analytical specificity of these assays was confirmed as all bacterial reference strains and clinical isolates tested were identified by their respective assays, with no non-specific detection observed (see Supplemental Tables 2.1, 2.2 and 2.3). The categorisation of *S. pneumoniae*, *N. meningitidis* and *H. influenzae* serogroups or serotypes, is based on unique polysaccharide capsule structure. Currently, 24 *S. pneumoniae* serotypes, 6 *N. meningitidis* serogroups and 6 *H. influenzae* serotypes are identified as the leading causes of bacterial meningitis infection [99]. In this study, these 6 *N. meningitidis* serogroups and 6 *H. influenzae* serotypes are all present in the bacterial reference strains evaluated (see Supplemental Table 2.2 and 2.3). Although all 24 *S. pneumoniae* serotypes are not present in the bacterial reference strains evaluated in this study, the *S. pneumoniae* *LepA* diagnostic target used, is a highly conserved protein present in all known bacterial genomes [142] and has previously been used as a *S. pneumoniae* diagnostic target [137]. The *N. meningitidis* and *H. influenzae* biomarkers used in this study were selected based on previous studies highlighting their diagnostic utility (Higgins, pers. comm.) [143].

The LOD of the *S. pneumoniae*, *N. meningitidis* and *H. influenzae* singleplex RPA assays was established to be 4.1, 8.5 and 3.9 genome copies per reaction, respectively, using Probit analysis (see Supplemental Table 2.6). These LODs compare favourably to various *S. pneumoniae*, *N. meningitidis* and *H. influenzae* real-time PCR assays which report analytical sensitivities in the range of 3 - 90 genome copies per reaction [123, 144, 145]. Also, the typical pathogen load found in CSF clinical samples, the “gold standard” specimen for bacterial meningitis diagnosis, is within the analytical sensitivity range of the singleplex RPA assays developed in this study [51].

The *S. pneumoniae*, *N. meningitidis* and *H. influenzae* singleplex RPA assays were further developed, creating internally controlled duplex RPA assays through the incorporation of an IAC template and corresponding IAC exo probe. The optimum concentration of IAC template for each assay was found to be  $2 \times 10^3$  copies (Figure 2.2). Criteria for determining this were based on identifying IAC template concentrations that produced optimum bacterial target detection, earliest onset times and highest fluorescent levels of resulting amplification curves, while maintaining efficient IAC detection. The robustness of each duplex RPA assay was subsequently demonstrated by the successful detection of 100 copies of respective bacterial targets, in the presence of 400 ng exogenous DNA, with the simultaneous detection of each

IAC (Figure 2.2). The presence of exogenous DNA in all three internally controlled duplex RPA assays caused minimal inhibition of bacterial target detection, resulting in slightly lower fluorescence levels and increased detection times. The exogenous DNA also slightly inhibited detection of the IAC in the *N. meningitidis* and *H. influenzae* assays, producing lower fluorescence levels. However, in this presence of high levels of background exogenous DNA, each bacterial target was successfully detected in under 10 min, demonstrating the efficient robustness of the optimised duplex RPA assays.

The clinical performance of all three optimised internally controlled duplex RPA assays was evaluated by testing 64 PCR-positive *S. pneumoniae*, *N. meningitidis* and *H. influenzae* clinical samples (see Supplemental Table 2.7) provided by the IMSRL. Resulting diagnostic sensitivity, or the proportion of confirmed-positive samples correctly identified as such, for the *S. pneumoniae*, *N. meningitidis* and *H. influenzae* internally controlled duplex RPA assays, was 100%, 85.7% and 100%, respectively. Resulting diagnostic specificity, or the proportion of confirmed-negative samples correctly identified as such, was 100% for all assays. The earliest detection times for clinical samples tested by the *S. pneumoniae*, *N. meningitidis* and *H. influenzae* duplex RPA assays was 5, 4.40 and 2.40 min, with average detection times of 7.20, 8.39 and 6.22 min, respectively (see Supplemental Table 2.7). Considering the parameters used in the IMSRL PCR evaluation, 1 cycle of 95°C for 20 s followed by 45 cycles of 95°C for 3 s and 60°C for 20 s, the resulting clinical sample detection times of the duplex RPA assays were significantly faster than the IMSRL PCR results. Clinical performance analysis of the internally controlled duplex RPA assays was evaluated by comparison to real-time PCR assay because previous comparisons of nucleic acid amplification diagnostics to culture-based methods have proven difficult [123, 131]. The 3 *N. meningitidis* IMSRL PCR-positive samples not detected by the internally controlled duplex RPA assay was possibly due to low bacterial target concentrations present in each sample, which correlates with the high PCR-Ct values (>35 Ct) of the IMSRL evaluation (see Supplemental Table 2.7). The subsequent re-confirmation of these samples as positive, using an “in-house” *N. meningitidis* PCR assay, was perhaps due to improved analytical sensitivity of the single-target PCR assay compared to the internally controlled two-target RPA assay.

Clinical isolates and samples used in this study were collected from culture or PCR confirmed cases of bacterial meningitis infection by the GUH and IMSRL during routine diagnostic services and in accordance with protocols approved by respective ethical committees. Anonymised residual specimens positive for *S. pneumoniae*, *N. meningitidis* or *H. influenzae* were supplied for assay evaluation in this study. The Ethics Committee of the National University of Ireland, Galway deemed that ethical approval was not required for the evaluation of these samples as analysis for human DNA was not carried out.

The incorporation of an IAC into nucleic acid diagnostics ensures assay validity by confirming negative results are not due to poor assay performance, but due to the absence or undetectable levels of target [52]. In this study, the results of the clinical evaluation of the *S. pneumoniae*, *N. meningitidis* and *H. influenzae* internally controlled duplex RPA assays, were successfully validated by the detection of the IAC template in all events of no bacterial target detection (see Supplemental Table 2.7). The IAC was not detected during the evaluation of 7 *S. pneumoniae* and 8 *H. influenzae* clinical samples, possibly due to competitive amplification of high bacterial target concentrations present. These results correlate with the early IMSRL PCR Ct-values for each of these samples, indicating the presence of high bacterial target loads. The performance guidelines however, for IAC incorporation into clinical nucleic acid diagnostics, only requires amplification of the IAC in the event of targets not being detected [52].

Positive control reactions carried out in this study performed successfully during analytical specificity testing and clinical evaluation, as the 100 copy template concentrations of respective type strains tested for each assay were detected in all reactions. Also, all NTC reactions carried out in this study performed successfully, as no detection was observed in any reactions. Furthermore, the NTC reactions for the internally controlled duplex RPA assays, doubled as positive controls, as the IAC template was successfully detected in the TAMRA channel in all events of no bacterial target detection in the FAM channel, indicating a successful uninhibited reaction.

The three RPA assays developed and evaluated in this study are the first report and clinical evaluation of duplex RPA assays incorporating competitive internal controls for the detection of leading bacterial meningitis causative pathogens: *S. pneumoniae*,

*N. meningitidis* and *H. influenzae*. The future developmental goal for the diagnostic technology detailed in this study will involve incorporation with an integrated POC diagnostic device for bacterial meningitis diagnosis. This technology will be developed with the aim of meeting ASSURED criteria [146] - Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable, for application in disease-prevalent developing countries.

## 2.5 Supplementary data

<b>Supplemental Table 2.1: <i>Streptococcus pneumoniae</i> RPA assay specificity panel.</b>		
Organism	Strain <sup>a</sup>	RPA Result
<b>Inclusivity Panel</b>		
<i>Streptococcus pneumoniae</i> reference strains and clinical isolates		
<i>S. pneumoniae</i> (1, Type strain)	DSM 20566	+ <sup>b</sup>
<i>S. pneumoniae</i> (3)	DSM 14377	+
<i>S. pneumoniae</i> (5)	DSM 14378	+
<i>S. pneumoniae</i> (9V)	DSM 11865	+
<i>S. pneumoniae</i> (19F)	DSM 24048	+
<i>S. pneumoniae</i> (19F)	DSM 25971	+
<i>S. pneumoniae</i> (23F)	DSM 11866	+
<i>S. pneumoniae</i> (23F)	DSM 11868	+
Clinical Isolates	14 Isolates	+
<b>Exclusivity Panel</b>		
<i>Streptococcus</i> reference strains and closely related non- <i>Streptococcus</i> reference strains		
<i>S. agalactiae</i>	BCCM 15081	-
<i>S. agalactiae</i>	BCCM 15082	-
<i>S. agalactiae</i>	BCCM 15083	-
<i>S. agalactiae</i>	BCCM 15084	-
<i>S. agalactiae</i>	BCCM 15085	-
<i>S. agalactiae</i>	BCCM 15086	-
<i>S. agalactiae</i>	BCCM 15087	-
<i>S. agalactiae</i>	BCCM 15094	-
<i>S. agalactiae</i>	BCCM 15095	-
<i>S. anginosus</i>	DSM 20563	-
<i>S. australis</i>	DSM 15627	-
<i>S. bovis</i>	DSM 20480	-
<i>S. canis</i>	DSM 20715	-
<i>S. constellatus</i>	DSM 20575	-
<i>S. cristatus</i>	DSM 8249	-
<i>S. downei</i>	DSM 5635	-
<i>S. dysgalactiae</i> subsp. <i>equisimilis</i>	DSM 6176	-
<i>S. equi</i> subsp. <i>equi</i>	DSM 20561	-
<i>S. equinus</i>	DSM 20558	-
<i>S. gordonii</i>	DSM 6777	-
<i>S. infantis</i>	DSM 12492	-
<i>S. intermedius</i>	DSM 20573	-
<i>S. mitis</i>	DSM 12643	-
<i>S. mutans</i>	DSM 20523	-
<i>S. oralis</i>	DSM 20066	-
<i>S. parasanguinis</i>	DSM 6778	-
<i>S. peroris</i>	DSM 12493	-
<i>S. porcinus</i>	DSM 20725	-

<i>S. pseudopneumoniae</i>	DSM 18670	-
<i>S. pyogenes</i>	DSM 2072	-
<i>S. pyogenes</i>	DSM 20565	-
<i>S. salivarius</i>	DSM 20560	-
<i>S. salivarius</i>	DSM 20617	-
<i>S. sanguinis</i>	DSM 20567	-
<i>S. sinensis</i>	DSM 14990	-
<i>S. suis</i>	DSM 9682	-
<i>S. uberis</i>	DSM 20569	-
<i>S. vestibularis</i>	DSM 5636	-
<i>H. influenzae</i>	DSM 4690	-
<i>H. influenzae</i>	DSM 11121	-
<i>H. parainfluenzae</i>	DSM 8978	-
<i>H. haemolyticus</i>	CCUG 15312	-
<i>H. somnus</i>	CCUG 12839	-
<i>N. meningitidis</i>	DSM 10036	-
<i>K. pneumoniae</i>	DSM 30184	-
<i>P. aeruginosa</i>	DSM 50071	-
<i>E. coli</i>	DSM 30083	-
<i>E. faecalis</i>	DSM 20317	-
<i>C. albicans</i>	CBS 2700	-
<i>B. fragilis</i>	DSM 2151	-
<i>M. cattarhalis</i>	DSM 11994	-
<i>S. aureus</i>	DSM 346	-

<sup>a</sup>DSM = Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures; BCCM = Belgian Coordinated Collections of Microorganisms; CCUG = Culture Collection, University of Göteborg, Sweden; CBS = CBS-KNAW Fungal Biodiversity Centre  
<sup>b</sup> + = positive; - = negative.

<b>Supplemental Table 2.2: <i>Neisseria meningitidis</i> RPA assay specificity panel.</b>		
Organism	Strain <sup>a</sup>	RPA Result
<b>Inclusivity Panel</b>		
<i>Neisseria meningitidis</i> reference strains and clinical isolates		
<i>N. meningitidis</i> (A, type strain)	NCTC 10025	+ <sup>b</sup>
<i>N. meningitidis</i> (A)	DSM 10036	+
<i>N. meningitidis</i> (A)	NCTC 3372	+
<i>N. meningitidis</i> (A)	NCTC 3375	+
<i>N. meningitidis</i> (B)	ATCC 13090	+
<i>N. meningitidis</i> (C)	ATCC 13102	+
<i>N. meningitidis</i> (C)	DSM 15464	+
<i>N. meningitidis</i> (W-135)	NCTC 11203	+
<i>N. meningitidis</i> (X)	NCTC 10790	+
<i>N. meningitidis</i> (Y)	NCTC 10791	+
Clinical Isolates	8 Isolates	+
<b>Exclusivity Panel</b>		
<i>Neisseria</i> reference strains and closely related non- <i>Neisseria</i> reference strains		
<i>N. animalis</i>	DSM 23392	-
<i>N. animaloris</i>	DSM 21642	-
<i>N. bacilliformis</i>	DSM 23338	-
<i>N. canis</i>	DSM 18000	-
<i>N. caviae</i>	DSM 23336	-
<i>N. cuniculi</i>	DSM 21768	-
<i>N. dentiae</i>	DSM 19151	-
<i>N. elongata</i> subsp. <i>elongata</i>	DSM 17712	-
<i>N. elongata</i> subsp. <i>glycolytica</i>	DSM 23337	-
<i>N. elongata</i> subsp. <i>nitroreducens</i>	DSM 17632	-
<i>N. flavescens</i>	DSM 17633	-
<i>N. gonorrhoeae</i>	ATCC 19424	-
<i>N. gonorrhoeae</i>	DSM 9189	-

<i>N. lactamica</i>	DSM 4691	-
<i>N. macacae</i>	DSM 19175	-
<i>N. mucosa</i>	DSM 17611	-
<i>N. ovis</i>	DSM 18075	-
<i>N. perflava</i>	DSM 18009	-
<i>N. shayeganii</i>	DSM 22246	-
<i>N. sicca</i>	DSM 17713	-
<i>N. subflava</i>	DSM 17610	-
<i>N. wadsworthii</i>	DSM 22247	-
<i>N. weaveri</i>	DSM 17688	-
<i>N. zoodegmatidis</i>	DSM 21643	-
<i>H. influenzae</i>	DSM 4690	-
<i>H. parainfluenzae</i>	DSM 8978	-
<i>H. haemolyticus</i>	CCUG 15312	-
<i>H. somnus</i>	CCUG 12839	-
<i>S. pneumoniae</i>	DSM 20566	-
<i>S. pseudopneumoniae</i>	DSM 18670	-
<i>S. agalactiae</i>	BCCM 15081	-
<i>S. mitis</i>	DSM 12643	-
<i>K. pneumoniae</i>	DSM 30104	-
<i>P. aeruginosa</i>	DSM 50071	-
<i>E. coli</i>	DSM 30083	-
<i>E. faecalis</i>	DSM 20371	-
<i>S. aureus</i>	DSM 346	-
<i>C. albicans</i>	CBS 7200	-

<sup>a</sup> DSM = Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures; BCCM = Belgian Coordinated Collections of Microorganisms; NCTC = National Collection of Type Cultures; CCUG = Culture Collection, University of Göteborg, Sweden; ATCC = American Type Culture Collection; CBS = CBS-KNAW Fungal Biodiversity Centre.

<sup>b</sup> + = positive; - = negative.

<b>Supplemental Table 2.3: <i>Haemophilus influenzae</i> RPA assay specificity panel.</b>		
Organism	Strain <sup>a</sup>	RPA Result
<b>Inclusivity Panel</b>		
<i>Haemophilus influenzae</i> reference strains and clinical isolates		
<i>H. influenzae</i> (B, Type Strain)	DSM 4690	+ <sup>b</sup>
<i>H. influenzae</i> (A)	NCTC 8465	+
<i>H. influenzae</i> (B)	DSM 10001	+
<i>H. influenzae</i> (B)	DSM 11969	+
<i>H. influenzae</i> (B)	DSM 11970	+
<i>H. influenzae</i> (B)	DSM 24049	+
<i>H. influenzae</i> (C)	NCTC 8469	+
<i>H. influenzae</i> (D)	DSM 11121	+
<i>H. influenzae</i> (E)	NCTC 8472	+
<i>H. influenzae</i> (F)	DSM 10000	+
<i>H. influenzae</i> (aegyptius)	DSM 21187	+
Clinical Isolates	18 Isolates	+
<b>Exclusivity Panel</b>		
<i>Haemophilus</i> reference strains and closely related non- <i>Haemophilus</i> reference strains		
<i>H. avium</i>	DSM 18557	-
<i>H. actinomycetemcomitans</i>	DSM 8324	-
<i>H. actinomycetemcomitans</i>	DSM 11122	-
<i>H. ducreyi</i>	DSM 8925	-
<i>H. ducreyi</i>	DSM 11479	-
<i>H. aphrophilus</i>	NCTC 11096	-
<i>H. haemolyticus</i>	NCTC 10839	-
<i>H. haemolyticus</i>	CCUG 15642	-
<i>H. haemolyticus</i>	CCUG 15312	-
<i>H. haemolyticus</i>	CDC-M 19501	-

<i>H. haemolyticus</i>	CCUG 12834	-
<i>H. haemolyticus</i>	CDC-M 21127	-
<i>H. haemolyticus</i>	CCUG 36015	-
<i>H. haemolyticus</i>	CCUG 24149	-
<i>H. parahaemolyticus</i>	DSM 21417	-
<i>H. pleuropneumoniae</i>	DSM13472	-
<i>H. paragallinarum</i>	DSM18554	-
<i>H. parainfluenzae</i>	DSM 8978	-
<i>H. parasuis</i>	DSM 21448	-
<i>H. pittmaniae</i>	DSM 21203	-
<i>H. pleuropneumoniae</i>	DSM 13472	-
<i>H. segnis</i>	NCTC 10977	-
<i>H. somnus</i>	CCUG12839	-
<i>H. felis</i>	DSM 21192	-
<i>S. pneumoniae</i>	DSM 11865	-
<i>S. pneumoniae</i>	DSM 11866	-
<i>S. aureus</i>	DSM 346	-
<i>K. pneumoniae</i>	DSM 30184	-
<i>M. cattarhalis</i>	DSM 11994	-
<i>C. albicans</i>	CBS 7200	-
<i>P. aeruginosa</i>	DSM 50071	-
<i>E. coli</i>	DSM 30083	-
<i>S. agalactiae</i>	BCCM 15081	-
<i>S. mutans</i>	DSM 20523	-
<i>B. fragilis</i>	DSM 2151	-
<i>S. epidermidis</i>	DSM 20044	-
<i>S. oralis</i>	DSM 20627	-
<i>S. pyogenes</i>	DSM 20565	-
<i>E. faecalis</i>	DSM 20317	-

<sup>a</sup>DSM = Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures; BCCM = Belgian Coordinated Collections of Microorganisms; NCTC = National Collection of Type Cultures; CCUG = Culture Collection, University of Göteborg, Sweden; CDC = Centre for Disease Control; CBS = CBS-KNAW Fungal Biodiversity Centre.

<sup>b</sup> + = positive; - = negative.

Supplemental Table 2.4: Additional RPA oligonucleotides evaluated	
Type	Sequence (5' - 3')
<i>S. pneumoniae</i>	
Forward Primers	CTCATCATGACAGCTCCGTCTGTTATTTACAAAG* <sup>Ta</sup> TCATGACAGCTCCGTCTGTTATTTACAAAGTTAA* <sup>T</sup> TCCGTCTGTTATTTACAAAGTTAATTTGACCGAC* <sup>G</sup> CTGTTATTTACAAAGTTAATTTGACCGACGGTGA* <sup>G</sup> ATTTACAAAGTTAATTTGACCGACGGTGAGTCTA* <sup>T</sup> CAAAGTTAATTTGACCGACGGTGAGTCTATGGAT* <sup>G</sup> TTAATTTGACCGACGGTGAGTCTATGGATGTGTC* <sup>T</sup> TTGACCGACGGTGAGTCTATGGATGTGTCTAACC* <sup>C</sup> CGACGGTGAGTCTATGGATGTGTCTAACCATCT* <sup>G</sup>
Reverse Primers	GCTCCGACGAACCTCCTGTGGTACCATGATTTGCG* <sup>C</sup> TTACTGCTCCGACGAACCTCCTGTGGTACCATGAT* <sup>T</sup> CTCCATTACTGCTCCGACGAACCTCCTGTGGTACC* <sup>A</sup> GCTAGCTCCATTACTGCTCCGACGAACCTCCTGTG* <sup>G</sup> GCTGAGCTAGCTCCATTACTGCTCCGACGAACCT* <sup>C</sup> CTTACGCTGAGCTAGCTCCATTACTGCTCCGACG* <sup>A</sup> CCACGCTTACGCTGAGCTAGCTCCATTACTGCTC* <sup>C</sup> CACAAAGTCCCCACGCTTACGCTGAGCTAGCTCC* <sup>A</sup> ATAGTCACAAAGTCCCCACGCTTACGCTGAGCTA* <sup>G</sup>
<i>N. meningitidis</i>	
Forward Primers	CTGCTCCAATTCCCGCACCCGAGCCACCGTATCA* <sup>C</sup> CCAATTCCCGCACCCGAGCCACCGTATCACCCA* <sup>C</sup> CCCGCACCCGAGCCACCGTATCACCACTCGTCT* <sup>G</sup>

Reverse Primers	CCCGAGCCACCGTATCACCCACTCGTCTGTGCTC*G CCACCGTATCACCCACTCGTCTGTGCTCGAAATA*G ATCACCCACTCGTCTGTGCTCGAAATAGGATAAA*G CCCACTCGTCTGTGCTCGAAATAGGATAAAGGCA*G CTGTGCTCGAAATAGGATAAAGGCAGGGAAAGCA*G CTCGAAATAGGATAAAGGCAGGGAAAGCAGATGC*G TTGGGCGGTTTTCGGACGTATCTGTTTGCA*C TTGTGTTGGGCGGTTTTCGGACGTATCTGTTTG*C TTTGAGATTGTGTTGGGCGGTTTTCGGACGTAT*C TCGCTGTTTGAGATTGTGTTGGGCGGTTTTCGGA*G TGGTGTGCTGTTTGAGATTGTGTTGGGCGGTTT*G TTGGTGGTGTGCTGTTTGAGATTGTGTTGGGCG*G TTTGTTGGTGGTGTGCTGTTTGAGATTGTGTTG*G TCGGTGGCTTTGTTGGTGGTGTGCTGTTTGA*G TGTGTTGTCGGTGGCTTTGTTGGTGGTGTGCT*G
<b><i>H. influenzae</i></b>	
Forward Primers	CAATTCACWACAGATCACACAATGGCGGGAACAT*C GGTCAATTCACWACAGATCACACAATGGCGGGAA*G ACTGGTCAATTCACWACAGATCACACAATGGCGG*G TTAAGTGGTCAATTCACWACAGATCACACAATGG*C CGCTTAACTGGTCAATTCACWACAGATCACACAA*T CAACGCTTAACTGGTCAATTCACWACAGATCACAC*G CTCAACGCTTAACTGGTCAATTCACWACAGATCAC*G TCACTCAACGCTTAACTGGTCAATTCACWACAGA*T ATAGGAGGGAAATGGTTATTACTTAAACCCAGCG*A CGCATAGGAGGGAAATGGTTATTACTTAAACCCA*G ATAACGCATAGGAGGGAAATGGTTATTACTTAAA*C CTGCATAACGCATAGGAGGGAAATGGTTATTACT*T TTTTCACCTGCATAACGCATAGGAGGGAAATGGT*G CAACTTTTTACCTGCATAACGCATAGGAGGGAA*A TTTCCAACCTTTTTACCTGCATAACGCATAGGAG*G CGTAATTTTTCCAACCTTTTTACCTGCATAACGCA*T TGTACGTAATTTTTCCAACCTTTTTACCTGCATAA*C
Reverse Primers	
** = phosphorothioate bond.	

<b>Supplemental Table 2.5: <i>Bacillus subtilis</i> DNA PCR-product internal amplification control (IAC) sequences for the duplex RPA assays (5' - 3').</b>
<b><i>Streptococcus pneumoniae</i> duplex RPA assay IAC</b>
TGGCGAGGCACTCGAGAGTCCCCACGCTTACGCTGAGCTAGCTCCATTACTCGTTGAATTTTTTCGATCATGC CCATCAGCAGCTTATGATCAATATGATCCAAACCGAGGCGGTCAACCTGAAGCCGCTCCAGCGCATTCTGAG AAATGTCGTCAAATTAACCTTTGTAAATAACAGACGGAGCTGTGAATTCTGCCTCGCCA
<b><i>Neisseria meningitidis</i> duplex RPA assay IAC</b>
TGGCGAGGCACTCGAGCGTCTGTGCTCGAAATAGGATAAAGGCAGGGAAAGCGTTGAATTTTTTCGATCATGC CCATCAGCAGCTTATGATCAATATGATCCAAACCGAGGCGGTCAACCTGAAGCCGCTCCAGCGCATTCTGAG AAATGTCCACAATCTCAAACAGCGACACCACCAACAAAGCCAGAATTCTGCCTCGCCA
<b><i>Haemophilus influenzae</i> duplex RPA assay IAC</b>
TGGCGAGGCACTCGAGCACCTGCATAACGCATAGGAGGGAAATGGTTATTACGTTGAATTTTTTCGATCATGC CCATCAGCAGCTTATGATCAATATGATCCAAACCGAGGCGGTCAACCTGAAGCCGCTCCAGCGCATTCTATT GATGTTCCCGCCATTGTGTGATCTGTTGTGAAGAATTCTGCCTCGCCA

<b>Supplemental Table 2.6: Limit of detection (LOD) Probit analysis for the <i>S. pneumoniae</i>, <i>N. meningitidis</i> and <i>H. influenzae</i> singleplex RPA assays.</b>			
Genome Copy Concentration Tested	Replicates Detected / Replicates Tested		
	<i>S. pneumoniae</i>	<i>N. meningitidis</i>	<i>H. influenzae</i>
8	12 / 12	12 / 12	12 / 12
7	12 / 12	12 / 12	12 / 12
6	12 / 12	10 / 12	12 / 12
5	12 / 12	11 / 12	12 / 12
4	12 / 12	8 / 12	12 / 12
3	9 / 12	7 / 12	10 / 12
2	8 / 12	6 / 12	7 / 12
1	8 / 12	4 / 12	7 / 12
<b>Genome copy LOD per reaction (95% probability)</b>	<b>4.1</b>	<b>8.5</b>	<b>3.9</b>

<b>Supplemental Table 2.7: Clinical evaluation of the <i>S. pneumoniae</i>, <i>N. meningitidis</i> and <i>H. influenzae</i> internally controlled duplex RPA assays using the IMSRL clinical samples.</b>									
Sample No.	Clinical Specimen <sup>a</sup>	IMSRL PCR (Ct Value)	RPA (min)	RPA Result					
				<i>S. pneumoniae</i>		<i>N. meningitidis</i>		<i>H. influenzae</i>	
				Sample	IAC	Sample	IAC	Sample	IAC
<b><i>S. pneumoniae</i> Clinical Samples</b>									
1	BLD	34.04	12.00	+ <sup>b</sup>	+	-	+	-	+
2	BLD	31.88	7.33	+	+	-	+	-	+
3	BLD	30.17	7.00	+	+	-	+	-	+
4	BLD	27.16	6.33	+	+	-	+	-	+
5	BLD	25.30	5.67	+	-	-	+	-	+
6	BLD	36.11	9.00	+	+	-	+	-	+
7	CSF	23.23	7.00	+	-	-	+	-	+
8	CSF	36.96	10.33	+	+	-	+	-	+
9	CSF	27.95	5.33	+	-	-	+	-	+
10	CSF	33.92	7.00	+	+	-	+	-	+
11	CSF	33.65	6.33	+	+	-	+	-	+
12	CSF	35.81	9.67	+	+	-	+	-	+
13	CSF	26.64	5.00	+	-	-	+	-	+
14	CSF	29.67	6.00	+	+	-	+	-	+
15	CSF	25.67	5.33	+	-	-	+	-	+
16	FLD	32.71	7.33	+	+	-	+	-	+
17	FLD	27.61	8.00	+	-	-	+	-	+
18	FLD	30.55	8.00	+	+	-	+	-	+
19	PLF	34.02	8.67	+	+	-	+	-	+
20	PLF	29.75	7.00	+	+	-	+	-	+
21	PLF	28.83	6.67	+	+	-	+	-	+
22	PLF	34.36	7.67	+	+	-	+	-	+
23	Knee FLD	25.27	6.00	+	-	-	+	-	+
<b><i>N. meningitidis</i> Clinical Samples</b>									
24	BLD	36.93	-	-	+	-	+	-	+
25	BLD	29.69	5.67	-	+	+	+	-	+
26	BLD	29.42	6.00	-	+	+	+	-	+
27	BLD	28.37	5.00	-	+	+	+	-	+
28	BLD	30.14	5.67	-	+	+	+	-	+
29	BLD	35.79	10.00	-	+	+	+	-	+
30	BLD	37.10	19.33	-	+	+	+	-	+
31	BLD	33.04	9.33	-	+	+	+	-	+
32	BLD	35.08	14.00	-	+	+	+	-	+
33	BLD	36.57	10.00	-	+	+	+	-	+
34	BLD	35.77	9.00	-	+	+	+	-	+

35	BLD	23.41	4.67	-	+	+	+	-	+
36	BLD	34.71	9.33	-	+	+	+	-	+
37	BLD	30.84	5.67	-	+	+	+	-	+
38	BLD	34.03	8.00	-	+	+	+	-	+
39	BLD	34.08	8.67	-	+	+	+	-	+
40	BLD	38.67	-	-	+	-	+	-	+
41	CSF	38.27	15.33	-	+	+	+	-	+
42	CSF	35.00	-	-	+	-	+	-	+
43	CSF	27.61	5.00	-	+	+	+	-	+
44	CSF	28.09	5.00	-	+	+	+	-	+
<b><i>H. influenzae</i> Clinical Samples</b>									
45	BLD	38.21	10.67	-	+	-	+	+	+
46	BLD	35.89	10.00	-	+	-	+	+	+
47	BLD	38.30	13.67	-	+	-	+	+	+
48	BLD	37.56	7.33	-	+	-	+	+	+
49	BLD	36.32	5.67	-	+	-	+	+	+
50	BLD	37.77	8.00	-	+	-	+	+	+
51	BLD	33.84	6.00	-	+	-	+	+	+
52	BLD	39.78	9.67	-	+	-	+	+	+
53	BLD	39.62	13.33	-	+	-	+	+	+
54	BLD	34.21	5.67	-	+	-	+	+	+
55	CSF	37.36	7.00	-	+	-	+	+	+
56	CSF	32.95	5.67	-	+	-	+	+	+
57	CSF	22.17	3.33	-	+	-	+	+	-
58	CSF	24.99	3.67	-	+	-	+	+	-
59	BC	16.55	3.00	-	+	-	+	+	-
60	BC	16.72	2.67	-	+	-	+	+	-
61	BC	15.51	2.67	-	+	-	+	+	-
62	BC	17.59	3.00	-	+	-	+	+	-
63	BC	15.06	3.00	-	+	-	+	+	-
64	PLF	22.36	3.33	-	+	-	+	+	-
<sup>a</sup> BLD = blood; CSF = cerebrospinal fluid; FLD = fluid; PLF = pleural fluid; BC = blood culture. <sup>b</sup> + = positive; - = negative									

<b>Supplemental Table 2.8: IMSRL PCR oligonucleotides.</b>	
Type	Sequence (5'-3')
<b><i>S. pneumoniae</i></b>	
Forward Probe	ACGCAATCTAGCAGATGAAGC (FAM)CTCCCTGTATCAAGCGTTTTTCGGCA(BHQ1) <sup>a</sup>
Reverse	TCGTGCGTTTTAATTCCAGCT
<b><i>N. meningitidis</i></b>	
Forward Probe	TTGTGTGGAAGTTTAATTGTAGGATGC (FAM)TCCTTCATCAGGCCCCAGCG(BHQ1)
Reverse	TCAGATTGTTGCCCTAAAGAGACA
<b><i>H. influenzae</i></b>	
Forward Probe	ATGGCGGGAACATCAATGA (FAM)CGGTAATTGGGATCCAT(BHQ1)
Reverse	ACGCATAGGAGGGAAATGGTT
<sup>a</sup> FAM, 6-carboxyfluorescein fluorophore; BHQ1, black hole quencher	

<b>Supplemental Table 2.9: "In-house" PCR oligonucleotides.</b>	
Type	Sequence (5'-3')
<b><i>S. pneumoniae</i></b>	
Forward Probe	CTCGTAAGCGTAAACTCCTTG (FAM)ACGCATGAAATCCATCGGATCAGTT(BHQ1) <sup>a</sup>

Reverse	CATACTCAAGACGCTGAGGA
<i>N. meningitidis</i>	
Forward	CGACATGTTTCGAACGTAATCTCC
Probe	(FAM)TATCGGGCAAAGCCAAATGCGAAG(BHQ1)
Reverse	ATTTCGGTGGCGCGTTT
<i>H. influenzae</i>	
Forward	GGTACGCACYACGGACAATATG
Probe	(FAM)AGCTCTTGGTTGCTCTCAATGGCA(BHQ1)
Reverse	CCTGATTTAGCYGCTCGATAACA
<sup>a</sup> FAM, 6-carboxyfluorescein fluorophore; BHQ1, black hole quencher	

### **Chapter 3**

## **Evaluation of an Internally Controlled Multiplex *Tth* Endonuclease Cleavage Loop-Mediated Isothermal Amplification (TEC-LAMP) Assay for the Detection of Bacterial Meningitis Pathogens**

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#### **Author Contributions**

OH designed the study, carried out all experimental work and drafted the manuscript. MC, TWB and RC were involved in the collection and analysis of clinical samples. All authors contributed to the review of the final manuscript.

## Abstract

Bacterial meningitis infection is a leading global health concern for which rapid and accurate diagnosis is essential to reduce associated morbidity and mortality. Loop-mediated isothermal amplification (LAMP) offers an effective low-cost diagnostic approach; however, multiplex LAMP is difficult to achieve, limiting its application. We have developed novel real-time multiplex LAMP technology, TEC-LAMP, using *Tth* endonuclease IV and a unique LAMP primer/probe. This study evaluates the analytical specificity, limit of detection (LOD) and clinical application of an internally controlled multiplex TEC-LAMP assay for detection of leading bacterial meningitis pathogens: *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*. Analytical specificities were established by testing 168 bacterial strains, and LODs were determined using Probit analysis. The TEC-LAMP assay was 100% specific, with LODs for *S. pneumoniae*, *N. meningitidis* and *H. influenzae* of 39.5, 17.3 and 25.9 genome copies per reaction, respectively. Clinical performance was evaluated by testing 65 archived PCR-positive samples. Compared to singleplex real-time PCR, the multiplex TEC-LAMP assay demonstrated diagnostic sensitivity and specificity of 92.3% and 100%, respectively. This is the first report of a single-tube internally controlled multiplex LAMP assay for bacterial meningitis pathogen detection, and the first report of *Tth* endonuclease IV incorporation into nucleic acid amplification diagnostic technology.

## 3.1 Introduction

Bacterial meningitis infection is caused by the hematogenous spread of human commensal bacteria to the central nervous system, resulting in inflammation of the meninges. Infection typically presents with the rapid onset of features including fever, headache, photophobia, nausea, vomiting and neck stiffness [91]. The approximate annual incident rate of bacterial meningitis infection varies, 1–2 cases per 100,000 people with a 25% mortality rate in developed countries, or 1000 cases per 100,000 people with a 50% mortality rate in disease-burdened developing regions [99]. If left untreated, bacterial meningitis can be fatal, and almost one in every two survivors are left with severe and long-term neurological sequelae [102, 103].

*Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* are leading etiological agents of bacterial meningitis infection, with *S. pneumoniae* and *N. meningitidis* responsible for over 80% of all incidents in regions with routine *H. influenzae* vaccination [96, 97, 100]. Despite this, *H. influenzae* related meningitis remains a serious threat, especially among children under 5 years of age in regions without routine immunization.[98] Although vaccination programs have significantly reduced infection rates associated with these pathogens [91, 97], *S. pneumoniae* and *N. meningitidis* vaccines have altered bacterial meningitis epidemiology. The most significant indirect result of these immunization programs has been bacterial serotype or serogroup replacement increasing the incidence of meningitis caused by non-vaccine or non-typeable strains [99].

The early diagnosis and treatment of bacterial meningitis infection significantly reduces associated disease burden [112]. Microscopic examination of cerebrospinal fluid (CSF), and bacterial culture of both CSF and blood samples, are conventional diagnostic approaches [99]. However, culture can take up to 24–72 h, with reduced sensitivity in cases of prior antimicrobial treatment, requiring initial administration of broad-spectrum antibiotics targeting multiple potential causative pathogens. This use of broad-spectrum empiric therapy can expose patients to adverse effects, increase costs and introduce selective pressure favoring the emergence and dissemination of antimicrobial resistance [113]. Delay in diagnosis of bacterial meningitis infection also prolongs the implementation of public health response, essential for reducing disease transmission. Conventional microbiological diagnostics are also inefficient for simultaneous pathogen identification in cases such as: co-infection; differentiation from other infectious diseases such as cryptococcal or tuberculous meningitis; and detection of non-culturable organisms in cases of prior antibiotic treatment [131, 147].

Nucleic acid diagnostic technologies offer advantages over conventional methods, with real-time polymerase chain reaction (PCR) being the most commonly employed approach [140]. Compared to traditional diagnostics, real-time PCR provides a faster, higher throughput, closed system technology, improved diagnostic sensitivity and specificity, and the ability to detect non-culturable pathogens [99, 139]. In addition, the simultaneous detection of multiple pathogens using multiplex real-time PCR reduces analysis time and reagent cost while also conserving clinical specimens [51]. However, real-time PCR requires expensive thermocycling equipment, making it a

less accessible diagnostic technology compared to traditional methods in settings with limited resources.

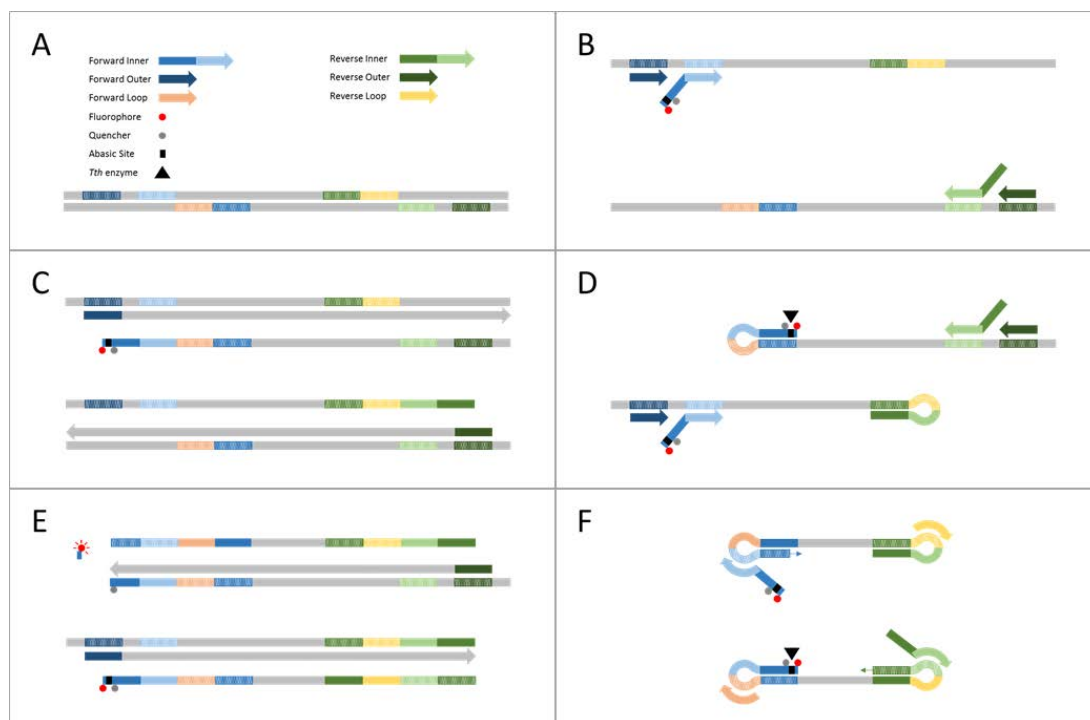
Loop-mediated isothermal amplification (LAMP), a rapid, highly sensitive and specific, isothermal nucleic acid amplification technique, offers a less expensive alternative to real-time PCR as it does not require thermocycling [73, 74]. LAMP has also demonstrated improved speed, diagnostic sensitivity and specificity, and tolerance of inhibitory substances when compared to PCR [84, 148, 149]. Furthermore, the utility of LAMP as an effective point-of-care (POC) technology for low-resource disease-burdened areas has been demonstrated using inexpensive, handheld thermal instrumentation with basic LED or smartphone monitoring devices, requiring low power sources [150-154].

LAMP is typically performed at 65 °C using forward and reverse inner, outer and loop oligonucleotide primers, in combination with a strand displacement DNA polymerase. Critically, the inner primers contain both sense and antisense target DNA sequences enabling loop structure formation, producing LAMP's unique rapid self-priming amplification [73, 74]. Multiplex LAMP detection however is difficult to achieve as the non-exonuclease activity of the polymerase enzyme is not compatible with standard nucleic acid hydrolysis probes [90]. This is a major limitation for the diagnostic application of LAMP as multiplex capabilities reduce analysis time/cost, conserve clinical specimens and enable incorporation of assay validation internal controls, essential for clinical diagnostics [52].

This study introduces *Tth* Endonuclease Cleavage (TEC)-LAMP, the first reported single-tube internally controlled multiplex LAMP technology with clinically relevant analytical sensitivity. TEC-LAMP was achieved by incorporating *Tth* endonuclease IV, a thermostable enzyme that cleaves abasic sites present in dsDNA, and a novel TEC primer/probe, with standard LAMP assay conditions. The TEC primer/probe, a modified LAMP forward inner primer incorporating an abasic site flanked by a 5' fluorophore and an internal thymine coordinated quencher (Table 3.1), acts as both a real-time monitoring probe and a standard LAMP inner primer. The TEC-LAMP mechanism, outlined in Figure 3.1, enables real-time monitoring and quantification, simultaneous detection of multiple targets and incorporation of internal amplification control (IAC) validation. We have successfully demonstrated the internally controlled

multiplex TEC-LAMP detection of *S. pneumoniae*, *N. meningitidis* and *H. influenzae*, and further evaluated this assay in terms of analytical specificity, limit of detection (LOD) and clinical performance.

<b>Table 3.1: TEC-LAMP oligonucleotides.</b>	
<b>Primer Type</b>	<b>Sequence (5'-3')</b>
<b><i>S. pneumoniae</i></b>	
TEC primer/probe	(FAM)TGGA(dSpacer)AA(BHQ1-dT)GCTCTGGCTTTTGAAGTGA-CCTACACCAATATCCTCGCT
Forward Inner	TGGAAAATGCTCTGGCTTTTGAAGTGA-CCTACACCAATATCCTCGCT
Reverse Inner	TCTGTCTGGTAGACAGAATGACGGA-TCTTTGAGAATCAGATGCTGGA
Forward Outer	TCCGTCAACGAGGCACAA
Reverse Outer	AGCAAACCTACCAAGCGC
Forward Loop	TGATGAAACAGACAAGCTGATTCT
Reverse Loop	GCGCAATGATGGTATAATCCAG
<b><i>N. meningitidis</i></b>	
TEC primer/probe	(Cy5)TGTC(dSpacer)G(BHQ2-dT)GGCTTTGTTGGTGGTGTCCG-GTGCAAACAGATACGTCCG
Forward Inner	TGTCGGTGGCTTTGTTGGTGGTGTCCG-GTGCAAACAGATACGTCCG
Reverse Inner	CCGATGTACCAGCACCTTGTCC-GTTTGGCGCTGATTACGCCTC
Forward Outer	CCCAATTCCACATCAATACGTG
Reverse Outer	GTGGTGTCCGGTGGTGTG
Forward Loop	GAGATTGTGTTGGGCGGTTTG
Reverse Loop	CACCACTTGGAAAACAGAGGC
<b><i>H. influenzae</i></b>	
TEC primer/probe	(HEX)TGCC(dSpacer)C(BHQ1-dT)GCTTCACGTAAATTATTTGG-TGCTTATTCCTATCGTGGTACG
Forward Inner	TGCCGCTGCTTCACGTAAATTATTTGG-TGCTTATTCCTATCGTGGTACG
Reverse Inner	CTTGGTTGCTCTCAATGGCAAG-GCACGCCAGTTAAAATCCCT
Forward Outer	GGCTGGAGCATTCGCATT
Reverse Outer	TTCTCCTGAAATTCGGGCAA
Forward Loop	AACATATTGTCCGTAGTGCG
Reverse Loop	TGATGATTTGTTATCGAGCAGC
<b>Internal Amplification Control</b>	
TEC primer/probe	(CYAN)TGTT(dSpacer)A(BHQ1-dT)ATCCGCGATCCTTGCGTTGT-TCCCCGCTATGGAAGGTC
Forward Inner	TGTTTATATCCGCGATCCTTGCGTTGT-TCCCCGCTATGGAAGGTC
Reverse Inner	CACCTGTTCGTGTCGTATCGGT-ATGCATTACCAGAGTGCTCC
Forward Outer	TACAGCGAAAAGCCCAGC
Reverse Outer	AAGCGACGAATGTCCTGTG
Forward Loop	TCTTAATTGCTTGCCGGAGC
Reverse Loop	GAGCCATGTGCCATACTCGTC
FAM, 6-carboxyfluorescein fluorophore; dSpacer, 1',2'-dideoxyribose; BHQ1-dT, black hole quencher 1 linked to thymine; -, separation between 5' antisense and 3' sense inner primer sequences; Cy5, cyanine fluorophore; BHQ2-dT, black hole quencher 2 linked to thymine; HEX, 6-hexachlorofluorescein fluorophore; CYAN, LC@CYAN 500 fluorophore.	



**Figure 3.1: TEC-LAMP mechanism.** (A) TEC-LAMP oligonucleotide components, *Tth* endonuclease IV enzyme and dsDNA template with oligonucleotide targets highlighted. (B) Temperature enabled dsDNA dissociation followed by primer and TEC primer/probe hybridization to corresponding targets. (C) Inner primer strand displacement extension, via *Bst* polymerase, forms dsDNA. Outer primer strand displacement extension dissociates this newly formed dsDNA, forming inner primer linked ssDNA. (D) The complementary sections of this newly formed inner primer linked ssDNA hybridize, forming loop structures. The abasic site of the TEC primer/probe is now in dsDNA form, and thus, cleaved by the *Tth* endonuclease IV enzyme. TEC primer/probe, inner and outer primers hybridize upstream of the stem loop structures. (E): Inner primer strand displacement extension forms dsDNA and displaces the downstream stem loop structures, fully dissociating the TEC primer/probe fluorophore and quencher, producing fluorescence. Outer primer strand displacement extension displaces the newly formed dsDNA, producing inner primer linked ssDNA. (F) The complementary sections at each end of the newly formed inner primer linked ssDNA hybridize and form loop structures. These double looped DNA templates are targeted by the TEC primer/probe, inner and loop primers, leading to rapid self-primed exponential amplification with increased cleavage and fluorescence events.

## 3.2 Materials and methods

### 3.2.1 Bacterial strains, DNA isolation and quantification

A total of 168 bacterial reference strains and clinical isolates were evaluated in this study (Supplemental Table 3.1). Clinical isolates were collected from culture-confirmed cases of bloodstream infection as part of routine diagnostic service at Galway University Hospital (GUH). All strains, stored at  $-80\text{ }^{\circ}\text{C}$ , were cultured in brain heart infusion (BHI) media (Oxoid, Hampshire, UK) and incubated at  $37\text{ }^{\circ}\text{C}$  for

18 h, under microaerophilic conditions, excluding *Haemophilus* strains which were cultured using *Haemophilus* test media (Oxoid). Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) and quantified using the Qubit dsDNA broad range/high sensitivity assay kits and Qubit 2.0 fluorometer (Life Technologies, Warrington, UK), according to manufacturer's instructions. Resulting DNA concentrations were converted to genome copy values using genome size standards of 2.1 Mb, 2.2 Mb and 1.83 Mb for *S. pneumoniae*, *N. meningitidis* and *H. influenzae*, respectively.

### 3.2.2 Diagnostic targets and TEC-LAMP oligonucleotides

*SPNA45\_01710*, a chromosomal heparinase III-like protein gene (position 1743404-1743847 of accession number HE983624.1), and *NMO\_1242*, a chromosomal cytolysin secretion ABC transporter gene (position 1295659-1297887 of accession number AM889136.1), were identified as novel diagnostic targets for *S. pneumoniae* and *N. meningitidis*, respectively. Alignment analysis of whole genome sequences retrieved from the National Center for Biotechnology Information (NCBI) database identified these biomarkers as highly conserved orthologous regions. The *pstA* gene was previously identified as an effective diagnostic target for *H. influenzae* [155], and the IAC template was a 500 bp random DNA gBlocks<sup>®</sup> Gene Fragment (Supplemental Table 3.4) purchased from Integrated DNA Technologies (Leuven, Belgium). These targets were used to design TEC-LAMP oligonucleotides (Table 3.1) using PrimerExplorer V4 (Eiken Chemical). Standard desalted oligonucleotide primers were synthesized by Integrated DNA Technologies. TEC primer/probes for *S. pneumoniae*, *N. meningitidis*, *H. influenzae* and the IAC, labeled with FAM, Cy5, HEX and Cyan fluorophores, respectively, were HPLC purified and synthesized by Metabion International AG (Planegg, Germany). Each fluorophore corresponded to one of four detection channels of the LightCycler<sup>®</sup> 480 instrument II (Roche Diagnostics, Sussex, UK) used to perform the LAMP reactions.

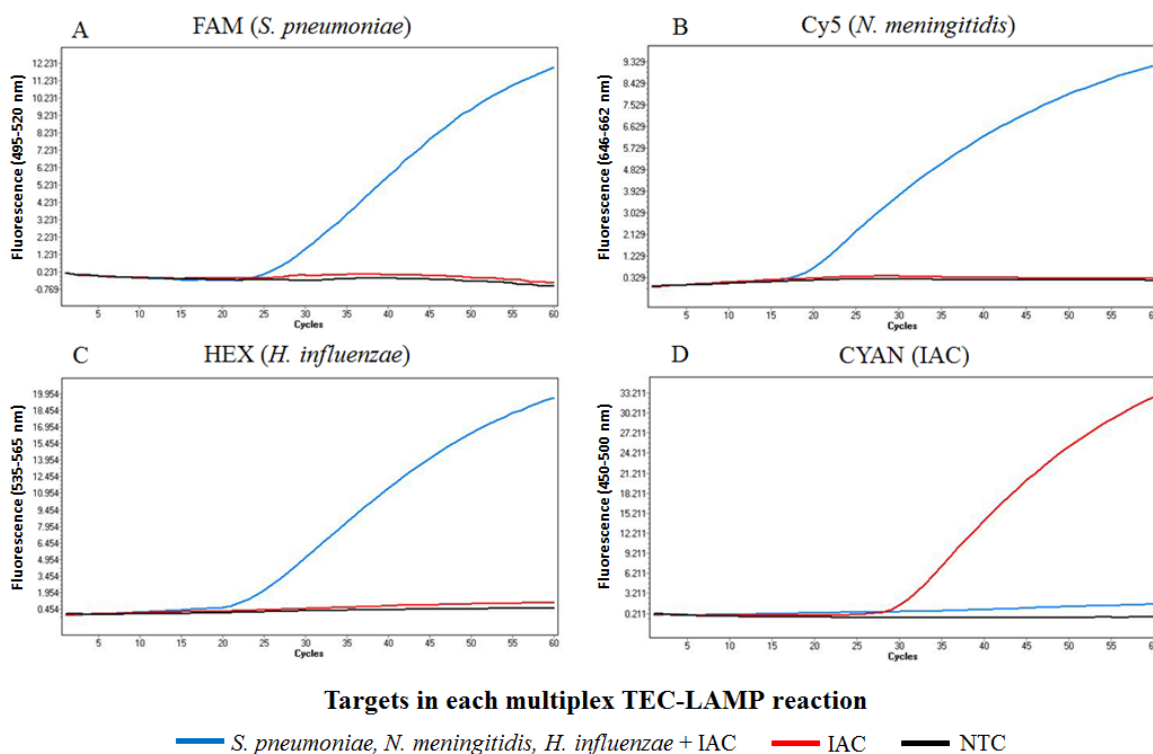
### 3.2.3 Internally controlled multiplex TEC-LAMP assay

TEC-LAMP oligonucleotide mixes consisting of 20  $\mu\text{M}$  reverse inner, 10  $\mu\text{M}$  forward inner and TEC primer/probe, 5  $\mu\text{M}$  forward and reverse loop, and 2.5  $\mu\text{M}$  forward and reverse outer, were prepared for each target and varying volumes were added to the final multiplex TEC-LAMP reaction: 3.25  $\mu\text{L}$  *S. pneumoniae*, 1.25  $\mu\text{L}$  *N. meningitidis*, 1.5  $\mu\text{L}$  *H. influenzae* and 0.75  $\mu\text{L}$  IAC. The final TEC-LAMP reaction contained 1 $\times$  Isothermal Amplification Buffer (New England Biolabs, Hitchin, UK), 6 mM  $\text{MgSO}_4$  (Roche Diagnostics), 1.4 mM deoxynucleotide triphosphate set (New England Biolabs), *S. pneumoniae* oligonucleotides [2.6  $\mu\text{M}$  reverse inner, 1.3  $\mu\text{M}$  forward inner and TEC primer/probe, 0.65  $\mu\text{M}$  forward and reverse loop, 0.325  $\mu\text{M}$  forward and reverse outer], *N. meningitidis* oligonucleotides [1  $\mu\text{M}$  reverse inner, 0.5  $\mu\text{M}$  forward inner and TEC primer/probe, 0.25  $\mu\text{M}$  forward and reverse loop, 0.125  $\mu\text{M}$  forward and reverse outer], *H. influenzae* oligonucleotides [1.2  $\mu\text{M}$  reverse inner, 0.6  $\mu\text{M}$  forward inner and TEC primer/probe, 0.3  $\mu\text{M}$  forward and reverse loop, 0.15  $\mu\text{M}$  forward and reverse outer], IAC oligonucleotides [0.6  $\mu\text{M}$  reverse inner, 0.3  $\mu\text{M}$  forward inner and TEC primer/probe, 0.15  $\mu\text{M}$  forward and reverse loop, 0.075  $\mu\text{M}$  forward and reverse outer], 8 U *Bst* 2.0 WarmStart DNA polymerase (New England Biolabs), 15 U *Tth* endonuclease IV (New England Biolabs), 1  $\mu\text{L}$  IAC template (50 copies), 1  $\mu\text{L}$  DNA template (1–3 templates) or 1  $\mu\text{L}$  molecular grade water for no template control (NTC) reactions, and molecular grade water to give a final volume of 25  $\mu\text{L}$ . Reactions were performed for 60  $\times$  1 min cycles at 67  $^\circ\text{C}$  in a LightCycler<sup>®</sup> 480 instrument II (Roche Diagnostics). The fluorescence detection channels used were 450–500 nm (Cyan), 495–520 nm (FAM), 535–565 nm (HEX) and 646–662 nm (Cy5), with fluorescent measurements recorded every cycle. A color compensation file, generated as per LightCycler<sup>®</sup> 480 operator manual, was applied for correction of any channel-to-channel fluorescence cross-talk.

### 3.2.4 Demonstration of internally controlled multiplex TEC-LAMP detection

For demonstration of the multiplex TEC-LAMP assay, type strain purified genomic DNA templates (*S. pneumoniae* DSM 20566, *N. meningitidis* NCTC 10025 and *H. influenzae* DSM 4690) were tested at 100 genome copy concentrations. Internally controlled multiplex TEC-LAMP detection was demonstrated by challenging the

assay with a combination of all three templates, in the presence of 50 copies IAC template. Control reactions with no bacterial template in the presence of 50 copies IAC template, and a NTC reaction, were performed in parallel. The LightCycler® 480 recorded positive results as exponential signal acquisition exceeding background fluorescence, represented as fluorescence amplification curves (Figure 3.2). Cycle threshold (Ct) values denoted cycles at which fluorescent signal exceeded background levels. As TEC-LAMP reactions were performed for  $60 \times 1$  min cycles on the LightCycler® 480 instrument II, resulting Ct-values acted as approximate time-to-positivity values in minutes.



**Figure 3.2: Internally controlled multiplex TEC-LAMP detection.** The four graphs show fluorescence recorded in the FAM (A), Cy5 (B), HEX (C) and CYAN (D) LightCycler® 480 detection channels, for three TEC-LAMP reactions performed in parallel. These reactions included: (blue) 100 genome copies *S. pneumoniae*, *N. meningitidis* and *H. influenzae* in the presence of 50 copies IAC; (red) no bacterial template in the presence of 50 copies IAC; and (black) a NTC reaction using molecular grade water in place of bacterial or IAC templates. Successful simultaneous detection of all three bacterial targets in the presence of the IAC was observed (blue: A–C). The two control reactions performed successfully as detection of the IAC in the absence of bacterial target was observed (Red: D), and no detection was observed in the NTC reaction (Black: A–D).

### 3.2.5 Analytical specificity and limit of detection

The analytical specificity of the TEC-LAMP assay was established by testing purified genomic DNA from a panel of bacterial strains (Supplemental Table 3.1) at  $10^5$  genome copy concentrations. The TEC-LAMP assay limit of detection (LOD) for *S. pneumoniae*, *N. meningitidis* or *H. influenzae*, in the presence of 50 copies IAC template, was established with 95% probability using Probit regression analysis. Type strain purified genomic DNA for each pathogen was tested using 6 replicates of 128, 64, 32, 16, 8 and 4 genome copy concentrations. Probit analysis was performed on the resulting data (Supplemental Table 3.2) using Minitab 17 (MiniTab, State College, PA, USA).

### 3.2.6 Clinical evaluation

TEC-LAMP clinical performance was evaluated by testing archived genomic DNA from PCR-confirmed cases of invasive pneumococcal, meningococcal and Haemophilus infection. All samples evaluated were supplied by the Irish Meningitis and Sepsis Reference Laboratory (IMSRL), Dublin, Ireland. The IMSRL extracted genomic DNA from anonymized residual clinical specimens, followed by real-time PCR analysis for *S. pneumoniae*, *N. meningitidis* and *H. influenzae*, as part of routine diagnostic service. IMSRL DNA extractions were carried out in 210  $\mu$ L volumes using a QIASymphony SP/AS instrument with QIAamp DSP DNA Blood Mini Kits (Qiagen), as per manufacturer instructions, for automated extractions in 50  $\mu$ L elution volumes. IMSRL PCR assays (Supplemental Table 3.5) were performed using standard reaction conditions targeting the *S. pneumoniae* *lytA*, *N. meningitidis* *ctrA* and *H. influenzae* *fucK* genes, testing 2.5  $\mu$ L of each sample. A total of 65 samples including 34 blood, 5 blood culture, 17 CSF, 5 pleural fluid, 1 knee fluid and 3 other body fluids, were supplied with corresponding real-time PCR Ct-values (Supplemental Table 3.3). For comparative purposes, the diagnostic sensitivity and specificity of the TEC-LAMP assay was also determined by testing 2.5  $\mu$ L of each of sample. Any samples not detected using the TEC-LAMP assay were re-tested with “in-house” singleplex real-time PCR assays (Supplemental Table 3.6) using standard reaction conditions, targeting the *S. pneumoniae* *lepA*, *N. meningitidis* *NMO\_1242* and *H. influenzae* *pstA* genes.

### 3.3 Results

#### 3.3.1 Demonstration of internally controlled multiplex TEC-LAMP detection

The TEC-LAMP assay successfully demonstrated the internally controlled multiplex detection of 100 genome copies of all three bacterial targets, *S. pneumoniae*, *N. meningitidis* and *H. influenzae*, in the presence of 50 copies IAC template (Figure 3.2A–C, blue). In this reaction, the IAC template was not detected during the simultaneous co-amplification of the three bacterial targets (Figure 3.2D, blue). The control reactions performed successfully, with TEC-LAMP detection of 50 copies IAC template in the absence of bacterial template observed (Figure 3.2D, red) and no amplification observed in the no template control (NTC) reaction (Figure 3.2A–D, black).

#### 3.3.2 Analytical specificity and limit of detection

The TEC-LAMP assay demonstrated 100% analytical specificity. All inclusivity panel strains were detected in their respective detection channels, *S. pneumoniae* (FAM), *N. meningitidis* (Cy5) and *H. influenzae* (HEX), with none of the exclusivity panel strains detected (Supplemental Table 3.1). The IAC performed successfully during exclusivity panel testing as it was detected in all events of no bacterial target amplification (Supplemental Table 3.1). The TEC-LAMP assay LOD, for the separate detection of *S. pneumoniae*, *N. meningitidis* or *H. influenzae* in the presence of 50 copies IAC template, was confirmed with 95% probability using Probit analysis to be 39.5, 17.3 and 25.9 genome copies per reaction, respectively (Supplemental Table 3.2). The IAC performed successfully during Probit analysis testing as it was detected in all reactions.

#### 3.3.3 Clinical evaluation

The IMSRL PCR-positive clinical samples that tested positive with the TEC-LAMP assay, were only identified in corresponding detection channels (Supplemental Table 3.3). Thus, samples positive for a single species were considered negative control samples for the remaining two species. In total, 60/65 positive samples (22/23 *S.*

*pneumoniae*, 22/22 *N. meningitidis* and 16/20 *H. influenzae*), and 0/130 negative samples (0/42 *S. pneumoniae*, 0/43 *N. meningitidis* and 0/45 *H. influenzae*), were successfully identified as such. The 5 IMSRL PCR-positive samples not detected by the TEC-LAMP assay, were subsequently re-confirmed as positive using “in-house” real-time PCR assays, producing similar high Ct-values (>35 Ct) to that of the IMSRL PCR Ct-values. Thus, compared to the IMSRL PCR results, the overall diagnostic sensitivity and specificity of the multiplex TEC-LAMP assay was 92.3% and 100%, respectively. The diagnostic sensitivities for each pathogen were 95.7% (*S. pneumoniae*), 100% (*N. meningitidis*), and 80% (*H. Influenzae*). The IAC performed successfully during clinical sample testing as it was detected in all events of no bacterial target amplification (Supplemental Table 3.3).

### 3.4 Discussion

Bacterial meningitis infection is responsible for significant global morbidity and mortality [91]. Rapid and accurate detection of bacterial meningitis pathogens is essential for effective treatment and lowering associated disease burden [112]. This study has evaluated the analytical specificity, limit of detection (LOD) and clinical application of a novel internally controlled multiplex TEC-LAMP assay for the detection of leading bacterial meningitis pathogens: *S. pneumoniae*, *N. meningitidis* and *H. influenzae*.

Analytical specificity of the TEC-LAMP assay was established to be 100%, as all bacterial strains tested were correctly identified in appropriate detection channels (Supplemental Table 3.1). This confirmed the utility of *SPNA45\_01710* and *NMO\_1242* genes as effective targets for *S. pneumoniae* and *N. meningitidis*, respectively. Various biomarkers for *S. pneumoniae* (*ply*, *lytA* and *Spn9802*) and *N. meningitidis* (*ctrA*, *sodC* and *siaD*) have been used for PCR and LAMP assay development [51, 84, 96, 123, 144, 148, 156, 157]; however, many of these targets have been identified as inefficient [123, 144, 155]. We propose that the *SPNA45\_01710* gene, and the sequence between bases 450-1800 of the *NMO\_1242* gene, are potential novel regions-of-interest for the identification of *S. pneumoniae* and *N. meningitidis*, respectively.

The TEC-LAMP assay LOD, established using Probit analysis (Supplemental Table 3.2), for *S. pneumoniae*, *N. meningitidis* or *H. influenzae* in the presence of 50 copies IAC template, was determined to be 39.5, 17.3 and 26.5 genome copies per reaction, respectively. Previously reported multiplex PCR assays and singleplex LAMP assays, for *S. pneumoniae*, *N. meningitidis* and *H. influenzae* identification, have shown similar analytical sensitivities of 3–60 and 10–100 genome copies per reaction, respectively [51, 84, 123, 144, 148, 156-158]. Also, the analytical sensitivity of the TEC-LAMP assay is within range of typical target pathogen loads in bacterial meningitis CSF specimens, the standard sample type for bacterial meningitis diagnosis [51].

Clinical application of the TEC-LAMP assay was evaluated by testing 65 anonymized residual samples that had previously tested PCR-positive for either *S. pneumoniae*, *N. meningitidis* or *H. influenzae* (Supplemental Table 3.3). The diagnostic sensitivity and specificity of the TEC-LAMP assay, defined as the proportion of confirmed-positive and confirmed-negative clinical samples correctly identified as such, was established to be 92.3% and 100%, respectively. Positive results were detected at an approximate average of 20 cycles (Supplemental Table 3.3) indicating an approximate average time-to-positivity of 20 min per sample. Considering the IMSRL PCR evaluation cycling parameters, 1 cycle of 95 °C for 20 s followed by 45 cycles of 95 °C for 3 s and 60 °C for 20 s, the resulting TEC-LAMP clinical sample time-to-positivity values were significantly faster than the IMSRL PCR results. As rapid bacterial meningitis diagnosis is essential for improved treatment and public health response [112], TEC-LAMP's faster time-to-detection results compared to real-time PCR highlight its diagnostic utility, especially for disease-burdened low-resource areas. Clinical performance comparison of the TEC-LAMP assay to the IMSRL PCR assays was chosen as previous studies have identified that comparing nucleic acid diagnostics to less sensitive culture methods can be problematic [123, 131]. Successful detection, using “in-house” PCR assays, of the 5 IMSRL PCR-positive samples that were not detected using TEC-LAMP, was possibly due to improved analytical sensitivity of the single-target PCR assays compared to the internally controlled multiple-target TEC-LAMP assay.

This study utilized clinical isolates/samples, from culture or PCR confirmed cases of bacterial meningitis infection, collected by the GUH and IMSRL during routine

diagnostic services and in accordance with respective ethical review committee approved protocols. Anonymized residual specimens positive for *S. pneumoniae*, *N. meningitidis* or *H. influenzae* were supplied for TEC-LAMP assay evaluation, and analysis for human DNA was not carried out. Considering this, the Ethics Committee of the National University of Ireland, Galway deemed that ethical approval for the evaluation of these samples was not required.

IAC incorporation into nucleic acid diagnostic assays provides validation by ensuring negative results are due to target absence and not assay performance [52]. The TEC-LAMP detection of IAC template in all events of no bacterial target amplification (Figure 3.2D, red; Supplemental Tables 3.1 and 3.3), successfully validated test results in this study. The events of no IAC detection during specificity testing, clinical testing and the demonstration of multiplex TEC-LAMP detection (Figure 3.2D, blue), were due to inhibition from amplification of bacterial targets. However, IAC performance guidelines for nucleic acid diagnostics only requires IAC detection in the event of respective targets not being detected [52].

The multiplex capability of TEC-LAMP to simultaneously detect multiple bacterial meningitis pathogens is essential as approximately 1% of all cases are a result of co-infection, such as *S. pneumoniae* with *N. meningitidis* or *S. pneumoniae* with *H. influenzae* [131]. The TEC-LAMP assay successfully demonstrated the simultaneous detection of all three bacterial pathogens in the presence of 50 copies IAC template (Figure 3.2A–C, blue). In this reaction, the IAC was not detected (Figure 3.2D, blue) due to inhibition from co-amplification of the three bacterial targets. However, this is the result of the IAC template being present at a low copy number (50 copies), and the IAC TEC-LAMP oligonucleotides being present at lower concentrations relative to the bacterial target TEC-LAMP oligonucleotides, to favor bacterial target detection. Both control reactions in this experiment performed successfully. The IAC template was detected in the absence of bacterial targets (Figure 3.2D, red) indicating a successful uninhibited reaction. Also, the no template control (NTC) reaction, incorporating molecular grade water in place of bacterial or IAC templates, performed successfully (Figure 3.2A–D, black) as no amplification was observed. This result demonstrated that the TEC-LAMP assay does not produce false positive results via oligonucleotide cross-reactivity in the absence of bacterial or IAC templates.

Performance or cost limitations of existing bacterial meningitis diagnostic methods has created demand for a rapid, sensitive, specific, low-cost, multiplex molecular diagnostic technology. To address this demand, multiplex LAMP methodologies have been reported; however, all approaches to date possess various limitations. Restriction enzyme gel electrophoresis [159], lateral flow biosensors [160] and pyrosequencing methods [161] have been used to demonstrate multiplex LAMP technology. These approaches however require laborious, contamination-prone, post-amplification analysis, preventing real-time quantifiable detection [162]. Multiplex LAMP based on antibody-antigen interactions has also been developed using immunochromatographic strips [163]. This approach however is difficult for non-culturable organisms and dependent on unique pathogen surface antigens. Turbidity monitoring [164] to achieve multiplex LAMP has also been reported; however, this approach has poor reproducibility and requires extensive design optimization to achieve differentiable time-to-positivity values.

Various closed-system multiplex LAMP methodologies have been reported. Tanner and colleagues developed a detection of amplification by release of quenching (DARQ) LAMP technology utilizing strand displacement of a quencher labeled inner primer hybridized to a shorter fluorophore labeled oligonucleotide [165]. This technology however inhibits the standard LAMP reaction, and is associated with non-template amplification and reduced time-to-positivity in multiplex format, major drawbacks for point-of-care (POC) or clinical applications. DARQ LAMP is also identical to a previously reported duplex LAMP method using “assimilating probe” technology [166]. This method however reported poor analytical sensitivity in both singleplex and duplex formats. QUASR LAMP [153, 154] is a multiplex LAMP technology with the converse labeling arrangement of DARQ LAMP, utilizing displacement of a fluorophore labeled inner primer hybridized to a shorter quencher labeled oligonucleotide. The fluorophore labeled inner primer is incorporated into the LAMP product and excess quencher labeled oligonucleotide suppresses any fluorescence from unincorporated inner primer. However, QUASR LAMP is limited to end-point detection and has only demonstrated duplex LAMP reactions. Also, due to the requirement of “excess” quencher labeled oligonucleotide, significantly increased oligonucleotide is required for each target compared to standard LAMP, thus increasing the possibility of non-specific detection. Wang and colleagues reported

MERT-LAMP, a multiplex technology using *Nb.BsrDI* endonuclease and an inner primer containing the *Nb.BsrDI* restriction site, flanked by a fluorophore and quencher [167]. This method however possesses a design limitation requiring the *Nb.BsrDI* restriction site to be absent from the diagnostic target to prevent unspecific cleavage disrupting the LAMP cycle. Also, addition of this restriction site sequence onto the inner primer significantly affects standard LAMP assay design, as well as  $T_m$  and G/C content values, increasing potential for inhibitory secondary structure formation.

Recently, Dou and colleagues detailed two separate multiplex LAMP methods for the detection of *S. pneumoniae*, *N. meningitidis* and *H. influenzae* through the use of polymer/paper hybrid biochips/spinchips [152, 168]. These methods however do not enable real-time detection, require expensive fluorescence microscopy to detect low-level target concentrations and do not enable internal control incorporation directly into the LAMP reaction chambers. Also, the NTC reactions reported by Dou and colleagues did not incorporate the LAMP primers present in the target reactions, presenting possible issues with reproducing effective signal differentiation between target and NTC reactions.

The only TEC-LAMP design limitation is requirement of a thymine residue, for quencher placement, to be in close proximity to the 5' fluorophore for sufficient quenching. This limitation however, can be overcome by coordinating the quencher to the adjacent sugar-phosphate primer backbone, instead of the thymine residue. TEC-LAMP does not create any additional design considerations to the standard LAMP method. Also, TEC-LAMP modifications cause minimal inhibition to the standard LAMP reaction (Supplemental Figure 3.1) indicating that incorporation of *Tth* endonuclease IV and the TEC primer/probe does not interfere with typical LAMP conditions. This also establishes that the cleaved 5' end of the TEC primer/probe can still hybridize to its complement (Figure 3.1F), maintaining loop structure formation capabilities after cleavage and confirming primer/probe properties. The multiplex TEC-LAMP method uses biased non-equimolar primer set concentrations, providing simplified assay optimization through varying the TEC-LAMP oligonucleotide mix volumes added to the final reaction. Optimization of these primer set mix volumes involves initially testing all sets at the same volume and subsequently adjusting these volumes based on assay performance, increased volumes for sub-optimal performing primer sets and decreased volumes for optimal performing primer sets. Relative inner,

outer and loop primer concentration ratios remain constant between primer sets, with a 50/50 ratio between the TEC primer/probe and forward inner primer. We observed that this 50/50 TEC primer/probe and forward inner primer ratio produced optimal results, while also reducing costs of using the more expensive TEC primer/probe as the only forward inner primer component. TEC-LAMP was evaluated on real-time PCR instrumentation; however this technology is compatible with cheaper multi-channel thermostatic fluorometers such as the Genie III<sup>®</sup> (OptiGene Ltd., Horsham, UK) or Twista<sup>®</sup> (TwistDx Ltd., Cambridge, UK). TEC-LAMP can also be applied to existing POC technology [150-154], such as simple heating devices combined with basic fluorescent detection methods.

The TEC-LAMP assay introduced in this study is the first report of a single-tube internally controlled multiplex LAMP assay with clinically relevant analytical sensitivity, and the first clinical evaluation of such technology applied to leading bacterial meningitis causative pathogens: *S. pneumoniae*, *N. meningitidis* and *H. influenzae*. This is also the first report of *Tth* endonuclease IV incorporation into nucleic acid amplification diagnostic technology. The TEC-LAMP methodology detailed in this study contributes to the current state-of-the-art in nucleic acid amplification diagnostics, providing novel transferable technology for infectious disease POC testing in low-resource disease-burdened areas.

### 3.5 Supplementary data

Table 3.1: Internally controlled multiplex TEC-LAMP assay specificity panel.					
Organism	Strain	TEC-LAMP Result			
		<i>S. pneumoniae</i> (FAM)	<i>N. meningitidis</i> (Cy5)	<i>H. influenzae</i> (HEX)	IAC (Cyan)
<b>Inclusivity Panel</b>					
<i>Streptococcus pneumoniae</i> Reference Strains and Clinical Isolates					
<i>S. pneumoniae</i> (1, Type strain)	DSM 20566	+	-	-	-
<i>S. pneumoniae</i> (3)	DSM 14377	+	-	-	-
<i>S. pneumoniae</i> (5)	DSM 14378	+	-	-	-
<i>S. pneumoniae</i> (9V)	DSM 11865	+	-	-	-
<i>S. pneumoniae</i> (19F)	DSM 24048	+	-	-	-
<i>S. pneumoniae</i> (19F)	DSM 25971	+	-	-	-
<i>S. pneumoniae</i> (23F)	DSM 11866	+	-	-	-
<i>S. pneumoniae</i> (23F)	DSM 11868	+	-	-	-
Clinical Isolates	14 Isolates	+	-	-	-
<i>Neisseria meningitidis</i> Reference Strains and Clinical Isolates					
<i>N. meningitidis</i> (A, type strain)	NCTC 10025	-	+	-	-
<i>N. meningitidis</i> (A)	DSM 10036	-	+	-	-

<i>N. meningitidis</i> (A)	NCTC 3372	-	+	-	-
<i>N. meningitidis</i> (A)	NCTC 3375	-	+	-	-
<i>N. meningitidis</i> (B)	ATCC 13090	-	+	-	-
<i>N. meningitidis</i> (C)	ATCC 13102	-	+	-	-
<i>N. meningitidis</i> (C)	DSM 15464	-	+	-	-
<i>N. meningitidis</i> (W)	NCTC 11203	-	+	-	-
<i>N. meningitidis</i> (X)	NCTC 10790	-	+	-	-
<i>N. meningitidis</i> (Y)	NCTC 10791	-	+	-	-
Clinical Isolates	9 Isolates	-	+	-	-
<i>Haemophilus influenzae</i> Reference Strains and Clinical Isolates					
<i>H. influenzae</i> (b, Type Strain)	DSM 4690	-	-	+	-
<i>H. influenzae</i> (a)	NCTC 8465	-	-	+	-
<i>H. influenzae</i> (b)	DSM 10001	-	-	+	-
<i>H. influenzae</i> (b)	DSM 11969	-	-	+	-
<i>H. influenzae</i> (b)	DSM 11970	-	-	+	-
<i>H. influenzae</i> (b)	DSM 24049	-	-	+	-
<i>H. influenzae</i> (c)	NCTC 8469	-	-	+	-
<i>H. influenzae</i> (d)	DSM 11121	-	-	+	-
<i>H. influenzae</i> (e)	NCTC 8472	-	-	+	-
<i>H. influenzae</i> (f)	DSM 10000	-	-	+	-
<i>H. influenzae</i> (aegyptius)	DSM 21187	-	-	+	-
Clinical Isolates	19 Isolates	-	-	+	-
<b>Exclusivity Panel</b>					
<i>Streptococcus</i> Reference Strains (excl. <i>S. pneumoniae</i> )					
<i>S. agalactiae</i>	BCCM 15081	-	-	-	+
<i>S. agalactiae</i>	BCCM 15082	-	-	-	+
<i>S. agalactiae</i>	BCCM 15083	-	-	-	+
<i>S. agalactiae</i>	BCCM 15084	-	-	-	+
<i>S. agalactiae</i>	BCCM 15085	-	-	-	+
<i>S. agalactiae</i>	BCCM 15086	-	-	-	+
<i>S. agalactiae</i>	BCCM 15087	-	-	-	+
<i>S. agalactiae</i>	BCCM 15094	-	-	-	+
<i>S. agalactiae</i>	BCCM 15095	-	-	-	+
<i>S. anginosus</i>	DSM 20563	-	-	-	+
<i>S. australis</i>	DSM 15627	-	-	-	+
<i>S. bovis</i>	DSM 20480	-	-	-	+
<i>S. canis</i>	DSM 20715	-	-	-	+
<i>S. constellatus</i>	DSM 20575	-	-	-	+
<i>S. cristatus</i>	DSM 8249	-	-	-	+
<i>S. downei</i>	DSM 5635	-	-	-	+
<i>S. dysgalactiae</i> subsp. <i>equisimilis</i>	DSM 6176	-	-	-	+
<i>S. equi</i> subsp. <i>equi</i>	DSM 20561	-	-	-	+
<i>S. equinus</i>	DSM 20558	-	-	-	+
<i>S. gordonii</i>	DSM 6777	-	-	-	+
<i>S. infantis</i>	DSM 12492	-	-	-	+
<i>S. intermedius</i>	DSM 20573	-	-	-	+
<i>S. mitis</i>	DSM 12643	-	-	-	+
<i>S. mutans</i>	DSM 20523	-	-	-	+
<i>S. oralis</i>	DSM 20066	-	-	-	+
<i>S. parasanguinis</i>	DSM 6778	-	-	-	+
<i>S. peroris</i>	DSM 12493	-	-	-	+
<i>S. porcinus</i>	DSM 20725	-	-	-	+
<i>S. pseudopneumoniae</i>	DSM 18670	-	-	-	+
<i>S. pyogenes</i>	DSM 2072	-	-	-	+
<i>S. pyogenes</i>	DSM 20565	-	-	-	+
<i>S. salivarius</i>	DSM 20560	-	-	-	+
<i>S. salivarius</i>	DSM 20617	-	-	-	+
<i>S. sanguinis</i>	DSM 20567	-	-	-	+
<i>S. sinensis</i>	DSM 14990	-	-	-	+

<i>S. suis</i>	DSM 9682	-	-	-	+
<i>S. uberis</i>	DSM 20569	-	-	-	+
<i>S. vestibularis</i>	DSM 5636	-	-	-	+
<i>Neisseria</i> Reference Strains (excl. <i>N. meningitidis</i> )					
<i>N. animalis</i>	DSM 23392	-	-	-	+
<i>N. animaloris</i>	DSM 21642	-	-	-	+
<i>N. bacilliformis</i>	DSM 23338	-	-	-	+
<i>N. canis</i>	DSM 18000	-	-	-	+
<i>N. caviae</i>	DSM 23336	-	-	-	+
<i>N. cuniculi</i>	DSM 21768	-	-	-	+
<i>N. dentiae</i>	DSM 19151	-	-	-	+
<i>N. elongata subsp. elongata</i>	DSM 17712	-	-	-	+
<i>N. elongata subsp. glycolytica</i>	DSM 23337	-	-	-	+
<i>N. elongata subsp. nitroreducens</i>	DSM 17632	-	-	-	+
<i>N. flavescens</i>	DSM 17633	-	-	-	+
<i>N. gonorrhoeae</i>	ATCC 19424	-	-	-	+
<i>N. gonorrhoeae</i>	DSM 9188	-	-	-	+
<i>N. gonorrhoeae</i>	DSM 9189	-	-	-	+
<i>N. lactamica</i>	ATCC 23970	-	-	-	+
<i>N. lactamica</i>	DSM 4691	-	-	-	+
<i>N. macacae</i>	DSM 19175	-	-	-	+
<i>N. mucosa</i>	DSM 17611	-	-	-	+
<i>N. ovis</i>	DSM 18075	-	-	-	+
<i>N. perflava</i>	DSM 18009	-	-	-	+
<i>N. polysaccharea</i>	DSM 22809	-	-	-	+
<i>N. shayeganii</i>	DSM 22246	-	-	-	+
<i>N. sicca</i>	DSM 17713	-	-	-	+
<i>N. subflava</i>	DSM 17610	-	-	-	+
<i>N. wadsworthii</i>	DSM 22247	-	-	-	+
<i>N. weaveri</i>	DSM 17688	-	-	-	+
<i>N. zoodegmatidis</i>	DSM 21483	-	-	-	+
<i>N. zoodegmatidis</i>	DSM 21643	-	-	-	+
<i>Haemophilus</i> Reference Strains (excl. <i>H. influenzae</i> )					
<i>H. actinomycetemcomitans</i>	DSM 8324	-	-	-	+
<i>H. actinomycetemcomitans</i>	DSM 11122	-	-	-	+
<i>H. aphrophilus</i>	NCTC 11096	-	-	-	+
<i>H. avium</i>	DSM 18557	-	-	-	+
<i>H. ducreyi</i>	DSM 8925	-	-	-	+
<i>H. ducreyi</i>	NCTC 11479	-	-	-	+
<i>H. equigenitalis</i>	DSM 10668	-	-	-	+
<i>H. felis</i>	DSM 21192	-	-	-	+
<i>H. haemoglobinophilus</i>	DSM 21241	-	-	-	+
<i>H. haemolyticus</i>	CCUG 12834	-	-	-	+
<i>H. haemolyticus</i>	CCUG 15312	-	-	-	+
<i>H. haemolyticus</i>	CCUG 15642	-	-	-	+
<i>H. haemolyticus</i>	CCUG 24149	-	-	-	+
<i>H. haemolyticus</i>	CCUG 34110	-	-	-	+
<i>H. haemolyticus</i>	CCUG 36015	-	-	-	+
<i>H. haemolyticus</i>	CCUG 36016	-	-	-	+
<i>H. haemolyticus</i>	CDC-M19501	-	-	-	+
<i>H. haemolyticus</i>	CDC-M21127	-	-	-	+
<i>H. haemolyticus</i>	CDC-M21621	-	-	-	+
<i>H. haemolyticus</i>	NCTC 10839	-	-	-	+
<i>H. paracuniculus</i>	DSM 21452	-	-	-	+
<i>H. paragallinarum</i>	DSM 18554	-	-	-	+
<i>H. parahaemolyticus</i>	DSM 21417	-	-	-	+
<i>H. parainfluenzae</i>	DSM 8978	-	-	-	+
<i>H. paraphrohaemolyticus</i>	DSM 21451	-	-	-	+
<i>H. parasuis</i>	DSM 21448	-	-	-	+
<i>H. pittmaniae</i>	DSM 17240	-	-	-	+

<i>H. pittmaniae</i>	DSM 21203	-	-	-	+
<i>H. segnis</i>	NCTC 10977	-	-	-	+
<i>H. somnus</i>	CCUG 12839	-	-	-	+
<i>H. vaginalis</i>	DSM 4944	-	-	-	+

DSM, Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures; BCCM, Belgian Coordinated Collections of Microorganisms; NCTC, National Collection of Type Cultures; CCUG, Culture Collection, University of Göteborg, Sweden; CDC, Centre for Disease Control; ATCC, American Type Culture Collection; +, positive; -, negative.

<b>Table 3.2: Limit of detection (LOD) Probit analysis for the internally controlled multiplex TEC-LAMP detection of <i>S. pneumoniae</i>, <i>N. meningitidis</i> or <i>H. influenzae</i> in the presence of 50 copies IAC template.</b>			
Genome copy concentration tested	Replicates detected / Replicates tested		
	<i>S. pneumoniae</i>	<i>N. meningitidis</i>	<i>H. influenzae</i>
128	6 / 6	6 / 6	6 / 6
64	6 / 6	6 / 6	6 / 6
32	5 / 6	6 / 6	6 / 6
16	4 / 6	6 / 6	4 / 6
8	1 / 6	2 / 6	2 / 6
4	0 / 6	1 / 6	0 / 6
<b>Genome copy LOD per reaction (95% probability)</b>	<b>39.5</b>	<b>17.3</b>	<b>25.9</b>

<b>Table 3.3: Internally controlled multiplex TEC-LAMP assay clinical samples tested.</b>							
Sample No.	Clinical Specimen	IMSRL PCR (Ct Value)	TEC-LAMP Result				
			TEC-LAMP (Ct Value)	<i>S. pneumoniae</i> (FAM)	<i>N. meningitidis</i> (Cy5)	<i>H. influenzae</i> (HEX)	IAC (Cyan)
<b><i>S. pneumoniae</i> Clinical Samples</b>							
1	BLD	34.04	17.94	+	-	-	+
2	BLD	31.88	22.54	+	-	-	+
3	BLD	30.17	20.08	+	-	-	+
4	BLD	27.16	25.40	+	-	-	+
5	BLD	25.30	39.57	+	-	-	+
6	BLD	36.11	53.32	+	-	-	+
7	CSF	23.23	24.61	+	-	-	+
8	CSF	36.96	-	-	-	-	+
9	CSF	27.95	14.27	+	-	-	-
10	CSF	33.92	21.42	+	-	-	+
11	CSF	33.65	23.54	+	-	-	+
12	CSF	35.81	27.17	+	-	-	+
13	CSF	26.64	15.59	+	-	-	-
14	CSF	29.67	22.59	+	-	-	+
15	CSF	25.67	17.13	+	-	-	-
16	FLD	32.71	21.91	+	-	-	+
17	FLD	27.61	18.20	+	-	-	-
18	FLD	30.55	26.53	+	-	-	+
19	PLF	34.02	18.80	+	-	-	+
20	PLF	29.75	21.74	+	-	-	+
21	PLF	28.83	18.97	+	-	-	+
22	PLF	34.36	18.71	+	-	-	+
23	Knee FLD	25.27	16.29	+	-	-	-
<b><i>N. meningitidis</i> Clinical Samples</b>							
24	BLD	36.93	21.45	-	+	-	+
25	BLD	29.69	13.63	-	+	-	-
26	BLD	29.42	16.01	-	+	-	+
27	BLD	28.37	11.14	-	+	-	-

28	BLD	30.14	15.21	-	+	-	-
29	BLD	35.79	20.09	-	+	-	+
30	BLD	28.11	11.14	-	+	-	-
31	BLD	37.10	20.51	-	+	-	+
32	BLD	33.04	22.75	-	+	-	+
33	BLD	35.08	18.59	-	+	-	+
34	BLD	36.57	20.70	-	+	-	+
35	BLD	35.77	22.70	-	+	-	+
36	BLD	23.41	11.24	-	+	-	-
37	BLD	34.71	20.58	-	+	-	+
38	BLD	30.84	18.75	-	+	-	+
39	BLD	34.03	13.85	-	+	-	+
40	BLD	34.08	19.63	-	+	-	+
41	BLD	38.67	34.18	-	+	-	+
42	CSF	38.27	33.23	-	+	-	+
43	CSF	35.00	35.85	-	+	-	+
44	CSF	27.61	9.56	-	+	-	-
45	CSF	28.09	9.95	-	+	-	-
<b><i>H. influenzae</i> Clinical Samples</b>							
46	BLD	38.21	-	-	-	-	+
47	BLD	35.89	55.00	-	-	+	+
48	BLD	38.30	-	-	-	-	+
49	BLD	37.56	24.55	-	-	+	+
50	BLD	36.32	21.19	-	-	+	+
51	BLD	37.77	27.28	-	-	+	+
52	BLD	33.84	23.80	-	-	+	+
53	BLD	39.78	-	-	-	-	+
54	BLD	39.62	-	-	-	-	+
55	BLD	34.21	21.35	-	-	+	+
56	CSF	37.36	23.30	-	-	+	+
57	CSF	32.95	28.38	-	-	+	+
58	CSF	22.17	12.61	-	-	+	-
59	CSF	24.99	12.60	-	-	+	-
60	BC	16.55	13.01	-	-	+	-
61	BC	16.72	15.15	-	-	+	-
62	BC	15.51	12.95	-	-	+	-
63	BC	17.59	12.93	-	-	+	-
64	BC	15.06	13.99	-	-	+	-
65	PLF	22.36	12.00	-	-	+	-

BLD, blood; CSF, cerebrospinal fluid; FLD, fluid; PLF, pleural fluid; BC, blood culture; +, positive; -, negative.

**Table 3.4: Internal amplification control (IAC) gBlocks® Gene Fragment nucleotide sequence (5' - 3').**

CGGCGCAGTGCTGCCGACAGCCGGGCATTGTCTTTGGGGCGTTATTCGAGGGCACC CGGACCTAACTTGTCCGGGAC  
CACC CGGGGTAGTCATCGGGCTTATACAGCGAAAAGCCAGCACCCGGCTCCCCGCTATGGAAGGTCATTAGCTCC  
GGCAAGCAATTAAGAACAACGCAAGGATCGCGGATATAAACAGAGAAACGGCCGAATACACCTGTTTCGTGTCGTAT  
CGGTAAATAGCCTCGCGGAGCCATGTGCCATACTCGTCTGCGGAGCACTCTGGTAATGCATATGGTCCACAGGACA  
TTCGTGCTTCCGGGTATGCGCTCTATGTGACGGTCTTTTGGCGCACAAATGCTCAGCACCATTTAAATTAGACCG  
ACTCCAGATCTGTAAGGTCCGCCACGCAGACGACAGCCCACGGAGACCACTGACCGATCTACCTGAACGGCGACCA  
TCTGTGTGGTACTGGGGCGGAGAGATAACTACGGTGCCGCTTAC

**Table 3.5: IMSRL PCR oligonucleotides.**

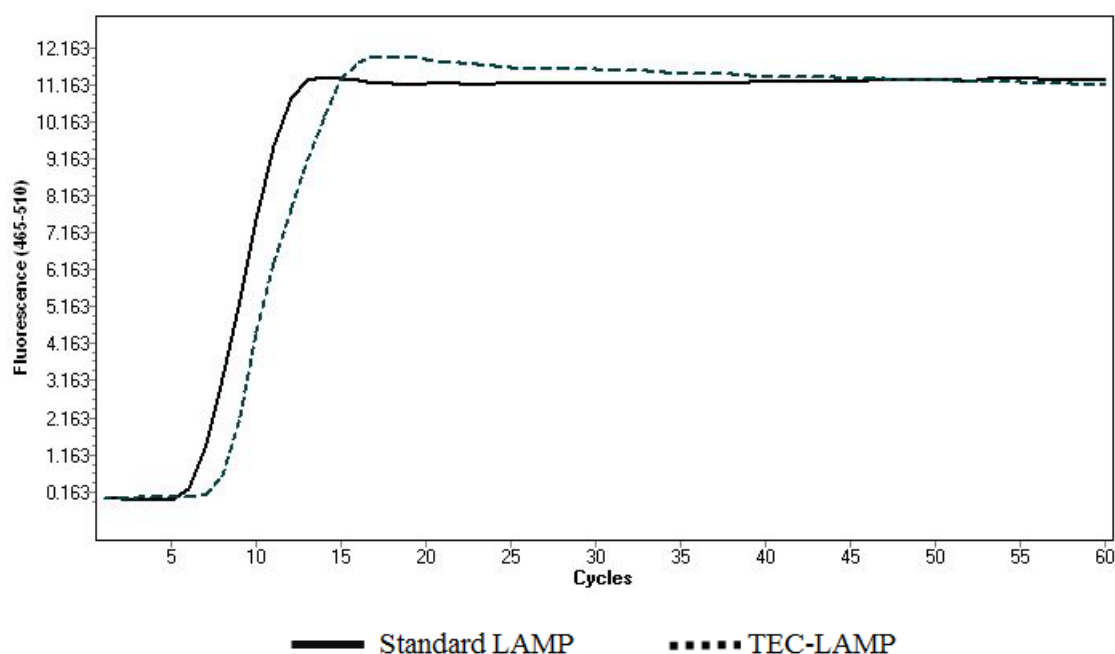
Type	Sequence
<i>S. pneumoniae</i>	
Forward	5'-ACGCAATCTAGCAGATGAAGC-3'
Probe	5'-(FAM)CTCCCTGTATCAAGCGTTTTTCGGCA(BHQ1)-3'

Reverse	5'-TCGTGCGTTTTAATTCCAGCT-3'
<i>N. meningitidis</i>	
Forward	5'-TTGTGTGGAAGTTTAATTGTAGGATGC-3'
Probe	5'-(FAM)TCCTTCATCAGGCCCCAGCG(BHQ1)-3'
Reverse	5'-TCAGATTGTTGCCCTAAAGAGACA-3'
<i>H. influenzae</i>	
Forward	5'-ATGGCGGGAACATCAATGA-3'
Probe	5'-(FAM)CGGTAATTGGGATCCAT(BHQ1)-3'
Reverse	5'-ACGCATAGGAGGGAAATGGTT-3'

FAM, 6-carboxyfluorescein fluorophore; BHQ1, black hole quencher

Table 3.6: "In-house" PCR oligonucleotides.	
Type	Sequence
<i>S. pneumoniae</i>	
Forward	5'-CTCGTAAGCGTAAACTCCTTG-3'
Probe	5'-(FAM)ACGCATGAAATCCATCGGATCAGTT(BHQ1)-3'
Reverse	5'-CATACTCAAGACGCTGAGGA-3'
<i>N. meningitidis</i>	
Forward	5'-CGACATGTTCGAACGTAATCTCC-3'
Probe	5'-(FAM)TATCGGGCAAAGCCAAATGCGAAG(BHQ1)-3'
Reverse	5'-ATTTCGGTGGCGCGTTT-3'
<i>H. influenzae</i>	
Forward	5'-GGTACGCACYACGGACAATATG-3'
Probe	5'-(FAM)AGCTCTTGGTTGCTCTCAATGGCA(BHQ1)-3'
Reverse	5'-CCTGATTTAGCYGCTCGATAACA-3'

FAM, 6-carboxyfluorescein fluorophore; BHQ1, black hole quencher



**Figure 3.1: Effect of TEC-LAMP modifications on standard LAMP reaction.** This experiment was carried out to determine if the TEC-LAMP modifications of oligonucleotide dye labels / abasic site and *Th* endonuclease IV enzyme, inhibit the standard unmodified LAMP reaction. The TEC-LAMP reaction was performed as previously described, however,

only the Cy5 labelled *N. meningitidis* primer set (Table 3.1) was used. For comparative purposes this reaction was performed with [dashed black] and without [solid black] the TEC-LAMP modifications. Both reactions were challenged with *N. meningitidis* DNA template at  $10^4$  copies. Reactions were performed for 60 cycles of 67°C for 1 min using a LightCycler 480 instrument II, with fluorescence recorded at each cycle in the FAM (495-520 nm) detection channel. As the Cy5 dye label on the *N. meningitidis* primer set is not detected in the FAM (495-520 nm) detection channel, 0.1X SYBR Green 1 intercalating dye was used to monitor the reaction. The resulting amplification curves indicate that the TEC-LAMP modifications cause minimal reaction inhibition to the standard unmodified LAMP reaction with detection observed only 2 cycles later than the unmodified LAMP reaction.

## **Chapter 4**

# **Loop-primer Endonuclease Cleavage Loop-Mediated Isothermal Amplification (LEC-LAMP) Technology for Singleplex or Multiplex Pathogen Detection with Flexible Single-Base Specificity for Single Nucleotide Polymorphism (SNP) Detection**

**Submitted for patent application with the Technology Transfer Office at the  
National University of Ireland, Galway**

## Abstract

Loop-mediated isothermal amplification (LAMP) provides effective infectious disease diagnostics technology, compatible with inexpensive instrumentation, for disease-prevalent developing regions. However, single nucleotide polymorphism (SNP) identification and simultaneous multiple-target detection, essential properties of efficient nucleic acid diagnostics, is difficult to achieve using LAMP. This study introduces LEC-LAMP, a singleplex or multiplex LAMP technology with single-base specificity for flexible SNP identification. We developed a singleplex LEC-LAMP *Neisseria meningitidis* assay that demonstrated complete analytical specificity with a limit of detection of 3.1 genome copies per reaction. Small scale clinical testing of this assay demonstrated 100% diagnostic specificity and sensitivity when assessed with anonymised DNA extracts from confirmed cases of bacterial meningitis infection. The single-base specificity of this assay indicated effective SNP identification properties when challenged with DNA templates containing SNPs located anywhere in a specific 6 base region. This assay was modified to create a SNP LEC-LAMP assay that successfully demonstrated single-tube wild-type and mutant allele differentiation. The singleplex assay was further modified to create a multiplex LEC-LAMP assay that successfully demonstrated simultaneously multiple-target detection of three bacterial targets, *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*, in a single reaction. LEC-LAMP is the first report of single-tube, real-time, singleplex or multiplex LAMP technology with single-base specificity for flexible SNP identification.

## 4.1 Introduction

Nucleic acid amplification technologies provide improved alternatives to conventional culture-based methods for the diagnosis of infectious diseases, in terms of diagnostic sensitivity and specificity, time-to-detection, reduced contamination and high throughput capabilities. Real-time polymerase chain reaction (PCR) is the benchmark nucleic acid diagnostic technology [32], however, for low-resourced disease-burdened regions it is an impractical point-of-care (POC) diagnostic option due to the requirement of expensive thermocycling equipment [169]. Isothermal nucleic acid amplification techniques do not require thermocycling and thus offer a more

convenient diagnostic option. Loop-mediated isothermal amplification (LAMP) is one of the most commonly used single-temperature nucleic acid amplification methods and has been extensively applied in the area infectious disease diagnostics [57, 170].

LAMP incorporates strand displacing *Bacillus stearothermophilus* (*Bst*) DNA polymerase with target-specific forward and reverse outer, inner and loop oligonucleotide primers. Typical LAMP reactions are performed at a single temperature ranging from 60-65°C, enabling initial target hybridisation by the inner and outer primers. Strand displacing primer extension combined with the sense and antisense inner primer sequences facilitates loop structure formation in LAMP, producing a unique double-looped DNA template. This template is targeted by the inner and loop oligonucleotide primers leading to rapid exponential target amplification. LAMP possesses high specificity due to the six target-specific primers recognising eight distinct regions on the target DNA, with single-digit genome copy sensitivity [73, 74]. Monitoring LAMP reactions can be performed using direct end-point visualisation or real-time turbidimetric analysis of magnesium pyrophosphate precipitation, a polymerisation by-product [79, 83]. Alternatively, post-amplification analysis or real-time monitoring of LAMP can be achieved using intercalating [84], colourimetric [85] or pH sensitive dyes [86]. LAMP is user-friendly, cost-effective, robust, capable of amplifying nucleic acid from samples without prior extraction and compatible with basic POC detection technologies [87-89], making LAMP an ideal near-patient diagnostic option.

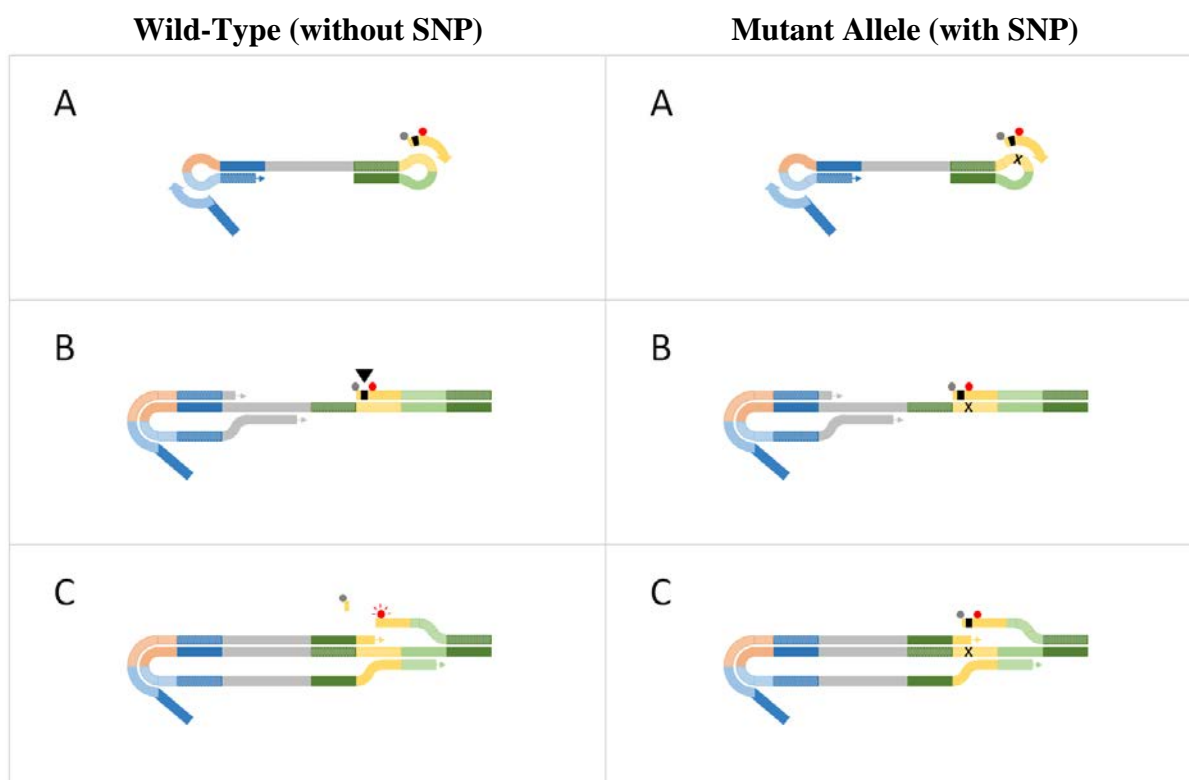
However, the non-exonuclease strand displacement activity of *Bst* DNA polymerase in LAMP is not compatible with standard nucleic acid hybridisation probes, making multiplex detection very difficult [90]. Clinical application of nucleic acid diagnostics requires multiplex detection capabilities for simultaneous pathogen detection, reduced analysis time, conservation of sample and incorporation of assay validating internal controls [51, 52]. In a recent study we introduced a novel internally controlled multiplex LAMP assay, *Tth* endonuclease cleavage loop-mediated isothermal amplification (TEC-LAMP), for the detection of bacterial meningitis associated pathogens [88]. TEC-LAMP is an adaptation of the standard LAMP method incorporating a restriction enzyme, *Tth* endonuclease IV, and a TEC primer/probe, an altered inner primer containing 5'-end modifications of an abasic site flanked by a fluorophore and quencher. These modifications enabled the development of a single-

tube internally controlled multiplex LAMP assay that could successfully detect multiple targets simultaneously. We identified, however, that the TEC-LAMP assay has limitations in terms of single-base specificity and cannot be utilised for single nucleotide polymorphism (SNP) identification.

A SNP is a single nucleotide sequence variation at a specific genome location, present in at least 1% of a population [27]. These are the simplest and most abundant form of genetic sequence variation occurring approximately once in every 1,000 bases [171]. Typically, SNPs are biallelic (two allele variants), with tri-allelic or tetra-allelic variants presenting less frequently [172], and are predominantly located in non-coding regions with minimal phenotypic impact. SNPs located in coding regions contribute to phenotypic variations, disease development and altered responses to drugs or environmental toxins [173]. Various SNPs are associated with cancer, cardiovascular disorders, diabetes, autoimmune diseases, gastrointestinal disorders and infectious diseases [174]. As a result, SNPs are commonly utilised as biomarkers for gene mapping and disease association studies, development of personalised medicines in pharmacogenetics, and molecular diagnostics [173]. DNA sequencing is widely used for SNP analysis, however, due to the requirement of extensive instrumentation and data analysis, this approach is more practical for SNP discovery instead of rapid POC application [89]. Nucleic acid infectious disease diagnostics utilise SNPs, and pathogen point-mutations associated with antimicrobial resistances, for accurate disease diagnosis facilitating improved treatment and reduced antimicrobial resistance dissemination [26, 175]. Nucleic acid diagnostic methods with SNP detection capabilities also enable greater specificity with effective differentiation of closely related pathogens. Typical nucleic acid SNP identification approaches involve differentiation of wild-type and mutant alleles using either allele-specific hybridisation or allele-specific enzymatic methods [171]. Combining SNP detection capabilities with rapid, sensitive and specific, multiplex isothermal nucleic acid techniques, such as TEC-LAMP, would provide very effective and transferable diagnostics technology for POC application.

This study introduces Loop-primer Endonuclease Cleavage Loop-Mediated Isothermal Amplification (LEC-LAMP). This novel technology improves on the single-base specificity limitation identified in the TEC-LAMP assay, through oligonucleotide and enzyme modifications. The TEC-LAMP 5'-end inner primer

modifications, involving an abasic site flanked by a fluorophore and quencher, are incorporated into the 5'-end of the loop primer in LEC-LAMP (Figure 4.1). In addition, the *Tth* endonuclease IV enzyme used in TEC-LAMP is replaced by endonuclease IV in LEC-LAMP. Using these modifications, we demonstrated single-target detection and SNP identification with a singleplex LEC-LAMP assay (one oligonucleotide primer set for one target) and compared this with a singleplex version of the previously reported TEC-LAMP technology. We further evaluated the singleplex LEC-LAMP assay in terms of analytical specificity, sensitivity and clinical application. Modified versions of this assay were used to demonstrate single-reaction differentiation of wild-type and mutant allele DNA templates using a SNP LEC-LAMP assay, as well as simultaneous multiple-target detection using a multiplex LEC-LAMP assay (multiple oligonucleotide primer sets for multiple targets). Leading pathogens associated with bacterial meningitis infection, *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*, were used as target pathogens to exemplify LEC-LAMP technology in this study.



**Figure 4.1: LEC-LAMP schematic.** (A) Loop regions of the double-looped LAMP template, produced by strand displacing polymerase extension from the outer and inner primers, are targeted by the inner primer and LEC primer/probe. (B) After primer and probe target hybridisation, strand displacement polymerase extension initiates, unwinding both loop

structures. LEC primer/probe target hybridisation produces a dsDNA abasic site initiating endonuclease IV cleavage in the wild-type reaction. Presence of the SNP in the mutant allele reaction inhibits abasic site dsDNA formation, preventing cleavage. (C) Combination of reaction temperature and strand displacement polymerase extension causes fluorophore and quencher dissociation in the wild-type reaction, producing fluorescence. In the mutant allele reaction, the fluorophore and quencher remain associated, preventing fluorescence production and enabling wild-type and mutant allele differentiation.

## 4.2 Materials and methods

### 4.2.1 Bacterial DNA template preparation

The singleplex LEC-LAMP *N. meningitidis* assay was evaluated using a range of *N. meningitidis*, *Neisseria* and closely related non-*Neisseria* reference strains (Supplemental Table 4.1). The multiplex LEC-LAMP *N. meningitidis*, *S. pneumoniae* and *H. influenzae* assay was performed using type-strains *N. meningitidis* NCTC 10025, *S. pneumoniae* DSM 20566 and *H. influenzae* DSM 4690. All bacterial strains, stored at -80°C, were cultured in brain heart infusion (BHI) media (Oxoid, Hampshire, UK) at 37°C for 18 h under microaerophilic conditions, excluding *Haemophilus* strains which were cultured using *Haemophilus* test media (Oxoid). DNA extractions were performed using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) followed by DNA quantification using the Qubit dsDNA broad range/high sensitivity assay kits and Qubit 2.0 fluorometer (Life Technologies, Warrington, UK). Genome size standards of 2.2 Mb, 2.1 Mb and 1.83 Mb for *N. meningitidis*, *S. pneumoniae* and *H. influenzae*, respectively, were used to convert resulting DNA concentrations to genome copy values. Extracted DNA samples were stored at -80°C prior to use.

### 4.2.2 Diagnostic targets and LEC-LAMP oligonucleotides

Previously evaluated *N. meningitidis*, *S. pneumoniae* and *H. influenzae* TEC-LAMP oligonucleotides [88], designed with PrimerExplorer V4 (Eiken Chemical) using diagnostic targets *NMO\_1242*, *SPNA45\_01710* and *pstA*, respectively, were modified to create the LEC-LAMP and TEC-LAMP oligonucleotides used in this study (Table 4.1). Oligonucleotide modifications included design of new reverse loop primers for the *S. pneumoniae* and *H. influenzae* assays, with addition of two 5'-end thymine residues to the forward loop primer of the *N. meningitidis* assay. Standard desalted oligonucleotides were synthesised by Integrated DNA Technologies. The LEC

primer/probes for *N. meningitidis*, *S. pneumoniae* and *H. influenzae*, labelled with FAM, HEX and Cy5 fluorophores, respectively, and the TEC primer/probe labelled with a FAM fluorophore, were HPLC purified and synthesised by Metabion International AG (Planegg, Germany). Each fluorophore corresponded to one of three detection channels of the LightCycler® 480 instrument II (Roche Diagnostics, Sussex, UK) used to perform the LEC-LAMP reactions.

<b>Table 4.1: LEC-LAMP and TEC-LAMP oligonucleotides.</b>	
<b>Primer Type</b>	<b>Sequence (5'-3')</b>
<b><i>N. meningitidis</i> LEC-LAMP</b>	
Forward Inner	TGTCGGTGGCTTTGTTGGTGGTGTTCGC-GTGCAAACAGATACGTCCG
Reverse Inner	CCGATGTACCAGCACCTTGTC-GTTTGCCTGATTACGCCTC
Forward Outer	CCCAATCCACATCAATACGTG
Reverse Outer	GTGGTGTTCGGTGGTGTG
LEC Primer/Probe Wild-Type	(BHQ1)TTGA(dSpacer)A(FAM-dT)TGTGTTGGGCGGTTTG
LEC Primer/Probe Mutant	(BHQ1)TTGA(dSpacer)C(HEX-dT)TGTGTTGGGCGGTTTG ( <b>SNP2 template specific</b> )
Reverse Loop	CACCACTTGGAAAACAGAGGC
<b><i>S. pneumoniae</i> LEC-LAMP</b>	
Forward Inner	TGGAAAATGCTCTGGCTTTTGAAGTGA-CCTACACCAATATCCTCGCT
Reverse Inner	TCTGTCTGGTAGACAGAAATGACGGA-TCTTTGAGAATCAGATGCTGGA
Forward Outer	TCCGTCAACGAGGCACAA
Reverse Outer	AGCAAACCTACCAAGCGC
Forward Loop	TGATGAAACAGACAAGCTGATTCT
LEC Primer/Probe	(BHQ1)ACTC(dSpacer)CA(HEX-dT)GCGCAATGATGGTATAATCC
<b><i>H. influenzae</i> LEC-LAMP</b>	
Forward Inner	TGCCGCTGCTTACGTAATATTTGG-TGCTTATTCCTATCGTGGTACG
Reverse Inner	CTTGGTTGCTCTCAATGGCAAG-GCACGCCAGTAAAAATCCCT
Forward Outer	GGCTGGAGCATTGCGATT
Reverse Outer	TTCTCCTGAAATTCGGGCAA
Forward Loop	AACATATTGTCCGTAGTGCG
LEC Primer/Probe	(BHQ2)TTGT(dSpacer)A(Cy5-dT)CGAGCAGCTAAATCAGGGA
<b><i>N. meningitidis</i> TEC-LAMP</b>	
TEC Primer/Probe	(FAM)TGTC(dSpacer)G(BHQ1-dT)GGCTTTGTTGGTGGTGTTCGC-GTGCAAACAGATACGTCCG
Forward Inner	TGTCGGTGGCTTTGTTGGTGGTGTTCGC-GTGCAAACAGATACGTCCG
Reverse Inner	CCGATGTACCAGCACCTTGTC-GTTTGCCTGATTACGCCTC
Forward Outer	CCCAATCCACATCAATACGTG
Reverse Outer	GTGGTGTTCGGTGGTGTG
Forward Loop	TTGAGATTGTGTTGGGCGGTTTG
Reverse Loop	CACCACTTGGAAAACAGAGGC
-, separation between 5' antisense and 3' sense inner primer sequences; BHQ1-dT, black hole quencher 1 linked to thymine; dSpacer, 1',2'-dideoxyribose; FAM, 6-carboxyfluorescein fluorophore; HEX, 6-hexachlorofluorescein fluorophore; Cy5, cyanine fluorophore.	

#### 4.2.3 Singleplex LEC-LAMP *N. meningitidis* assay single-target detection with comparison to TEC-LAMP

The singleplex LEC-LAMP *N. meningitidis* assay reaction contained 1×Isothermal Amplification Buffer (New England Biolabs, Hitchin, UK), 6 mM MgSO<sub>4</sub> (Roche Diagnostics), 1.4 mM deoxynucleotide triphosphate set (New England Biolabs), *N. meningitidis* oligonucleotides [1.6 µM forward and reverse inner, 0.4 µM LEC primer/probe wild-type and reverse loop, 0.2 µM forward and reverse outer], 8 U *Bst*

2.0 WarmStart DNA polymerase (New England Biolabs), 1 U endonuclease IV (New England Biolabs), 1  $\mu$ L DNA template or 1  $\mu$ L molecular grade water for NTC reaction, and molecular grade water to give a final reaction volume of 25  $\mu$ L. The singleplex TEC-LAMP *N. meningitidis* assay was prepared as per the LEC-LAMP assay, with modifications: the endonuclease IV enzyme was replaced with 15 U *Tth* endonuclease IV (New England Biolabs); the LEC primer/probe wild-type was replaced with unmodified forward loop primer; and 0.8  $\mu$ M of the forward inner primer was replaced with TEC primer/probe (Table 4.1). Reactions were performed for 60 x 1 min cycles at 67°C in a LightCycler® 480 instrument II (Roche Diagnostics). The fluorescence detection channel used was 495-520 nm (FAM) with fluorescent measurements recorded every min. Single-target detection using the singleplex LEC-LAMP and TEC-LAMP *N. meningitidis* assays was demonstrated by challenging both assays with  $10^3$  copies of type-strain *N. meningitidis* genomic DNA (Figure 4.2). No template control (NTC) reactions using molecular grade water in place of bacterial template were carried out in parallel. Positive results in each reaction were recorded on the LightCycler® 480 as exponential signal acquisition exceeding background fluorescence and represented as fluorescence amplification curves. Cycle threshold (Ct) values denoted cycles at which fluorescent signal exceeded background levels. As reactions were performed for 60 x 1 min cycles, resulting Ct-values acted as approximate time-to-positivity values in minutes.

#### 4.2.4 Singleplex LEC-LAMP *N. meningitidis* assay analytical specificity, sensitivity and clinical sample testing

Analytical specificity of the singleplex LEC-LAMP *N. meningitidis* assay was evaluated using genomic DNA from a panel of bacterial reference strains (Supplemental Table 4.1) at  $10^5$  genome copy concentrations. The limit of detection (LOD) of the singleplex LEC-LAMP *N. meningitidis* assay was determined by testing 6 replicates of 32, 16, 8, 4, 2 and 1 genome copy concentrations of type-strain *N. meningitidis* NCTC 10025 genomic DNA. Probit regression analysis was performed on the resulting data using Minitab 17 (Supplemental Table 4.2) to establish assay LOD with 95% probability. The clinical application of the singleplex LEC-LAMP *N. meningitidis* assay was assessed using archived genomic DNA extracted from blood

and cerebrospinal fluid (CSF) samples of confirmed bacterial meningitis cases. The Irish Meningitis and Sepsis Reference Laboratory (IMSRL) supplied 72 anonymised samples which were previously collected and processed as part of routine diagnostic service. IMSRL DNA extractions were carried out using a QIA Symphony SP/AS instrument with QIAamp DSP DNA Blood Mini Kits (Qiagen), as per manufacturer instructions, followed by real-time PCR analysis for *N. meningitidis*, *S. pneumoniae* and *H. influenzae*. We reconfirmed the presence of *N. meningitidis*, *S. pneumoniae* and *H. influenzae* in each respective sample using singleplex real-time PCR assays targeting the *N. meningitidis* *NMO\_1242*, *S. pneumoniae* *lepA* and *H. influenzae* *pstA* genes (Supplemental Table 4.3). PCR reactions were performed on a LightCycler® 480 II instrument, using the LightCycler® 480 Probes Master kit (Roche Diagnostics) as per manufacturer instructions, testing 2.5 µL of each sample (Supplemental Table 4.4). For comparative purposes, the diagnostic sensitivity and specificity of the singleplex LEC-LAMP *N. meningitidis* assay was also determined by testing 2.5 µL of each sample (Supplemental Table 4.4). Samples from cases of meningococcal infection were used to determine LEC-LAMP diagnostic sensitivity, and samples from cases of pneumococcal and Haemophilus infection were used to determine LEC-LAMP diagnostic specificity. Positive control reactions incorporating respective type-strain genomic DNA at 10<sup>3</sup> genome copies, and negative control reactions substituting molecular grade water for bacterial template, were carried out in parallel to the above reactions.

#### 4.2.5 Singleplex LEC-LAMP *N. meningitidis* assay SNP identification with comparison to TEC-LAMP

Templates used to demonstrate LEC-LAMP single-base specificity were synthetic 500 bp DNA gBlocks® Gene Fragments (Table 4.2 and Supplemental Table 4.5) purchased from Integrated DNA Technologies (Leuven, Belgium). Each template was based on a 500 bp sequence of the *N. meningitidis* *NMO\_1242* diagnostic target. SNP0 was an exact copy of this sequence and acted as a wild-type template for positive control reactions. SNP1-6 were incomplete copies of this sequence containing single-base mismatches in close proximity to the LEC primer/probe wild-type basic site, and acted as mutant allele test templates for the LEC-LAMP assay. SNPA contained



assay with the addition of using two fluorescence detection channels, 495-520 nm (FAM, wild-type) and 535-565 nm (HEX, mutant allele). A colour compensation file, generated as per LightCycler® 480 operator manual, was applied for correction of any channel-to-channel fluorescence crosstalk. A NTC reaction as previously described was carried out in parallel.

#### *4.2.7 Multiplex LEC-LAMP *N. meningitidis*, *S. pneumoniae* and *H. influenzae* assay simultaneous multiple-target detection*

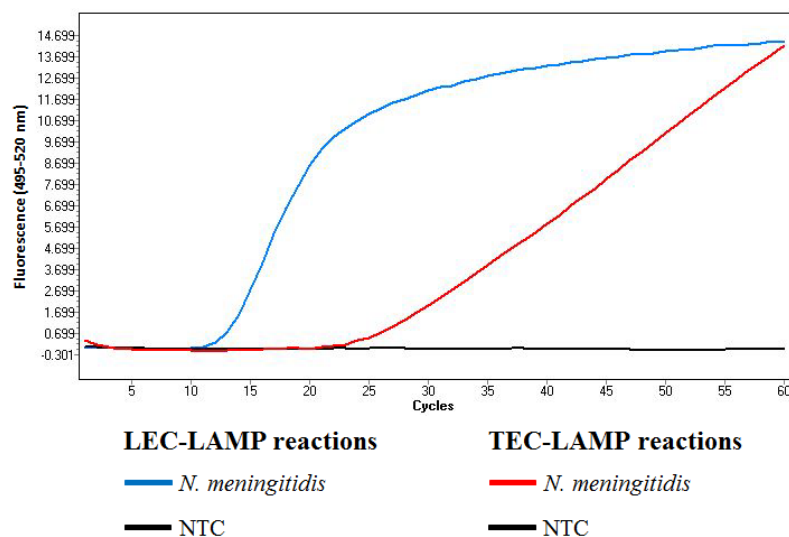
The multiplex LEC-LAMP *N. meningitidis*, *S. pneumoniae* and *H. influenzae* assay was prepared as per the singleplex LEC-LAMP *N. meningitidis* assay, with the further addition of *S. pneumoniae* and *H. influenzae* LEC-LAMP oligonucleotides (Table 4.1) at the same concentration as the *N. meningitidis* oligonucleotides. The addition of molecular grade water was altered to maintain a final reaction volume of 25 µL. Simultaneous multiple-target detection using the multiplex LEC-LAMP *N. meningitidis*, *S. pneumoniae* and *H. influenzae* assay was demonstrated by challenging the assay with *N. meningitidis* NCTC 10025, *S. pneumoniae* DSM 20566 and *H. influenzae* DSM 4690 purified genomic DNA at 10<sup>2</sup> genome copies, in a single reaction (Figure 4.5). This reaction was performed at 65°C using fluorescence detection channels 495-520 nm (FAM), 535-565 nm (HEX) and 646-662 nm (Cy5). A colour compensation file was generated as previously described and applied for correction of any channel-to-channel fluorescence crosstalk. A NTC reaction was carried out in parallel as previously described.

### **4.3 Results**

#### *4.3.1 Singleplex LEC-LAMP *N. meningitidis* assay single-target detection with comparison to TEC-LAMP*

Both the singleplex LEC-LAMP and TEC-LAMP *N. meningitidis* assays successfully demonstrated single-target detection of *N. meningitidis* at 10<sup>3</sup> genome copies (Figure 4.2, blue and red, respectively). However, the resulting amplification curves data highlighted significantly improved assay performance in the LEC-LAMP assay compared to the TEC-LAMP assay. The LEC-LAMP assay produced time-to-

positivity values of approximately 10 min compared to 20 min in the TEC-LAMP assay. Additionally, the LEC-LAMP assay produced higher fluorescence levels compared to the TEC-LAMP assay. The NTC reactions performed successfully as no amplification was observed (Figure 4.2, black).



**Figure 4.2: Singleplex LEC-LAMP *N. meningitidis* assay single-target detection with comparison to TEC-LAMP.** The singleplex LEC-LAMP (blue) and TEC-LAMP (red) *N. meningitidis* assays were challenged with *N. meningitidis* genomic DNA at  $10^3$  copies. No template control (NTC) reactions (black) were performed in parallel. Resulting LAMP fluorescence signal was recorded in the LightCycler® 480 FAM detection channel, with representative amplification curves for each reaction shown. Successful LEC-LAMP and TEC-LAMP detection of *N. meningitidis* is observed, however, LEC-LAMP produced earlier time-to-detection and increased fluorescence levels compared to TEC-LAMP. The NTC reactions performed successfully as no detection was observed.

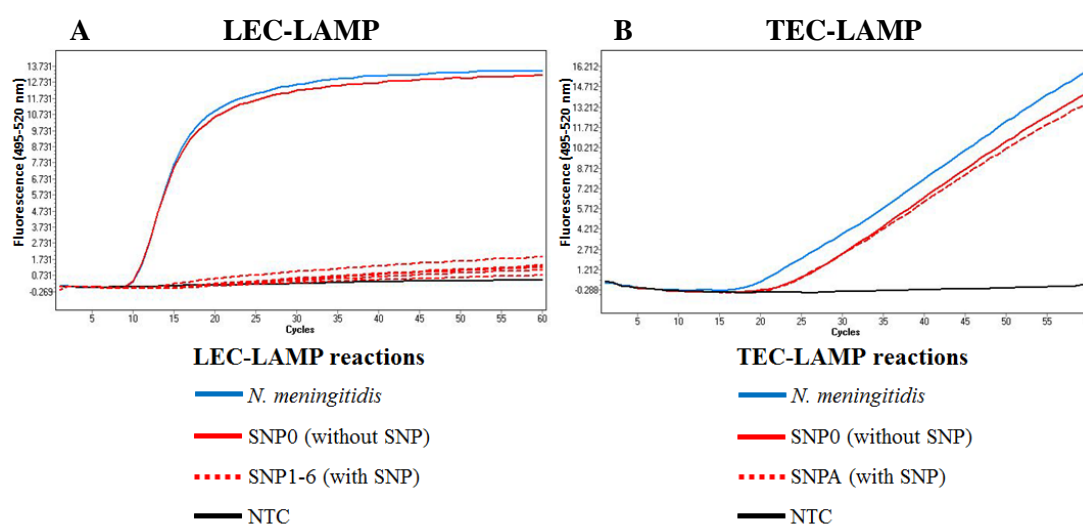
#### 4.3.2 Singleplex LEC-LAMP *N. meningitidis* assay analytical specificity, sensitivity and clinical sample testing

Complete analytical specificity was observed for the singleplex LEC-LAMP *N. meningitidis* assay as all *N. meningitidis* inclusivity panel reference strains were successfully detected, with no detection observed for the exclusivity panel reference strains (Supplemental Table 4.1). The limit of detection (LOD) for the singleplex LEC-LAMP *N. meningitidis* assay was confirmed with 95% probability using Probit analysis to be 3.1 genome copies per reaction (Supplemental Table 4.2). All IMSRL clinical samples provided, were successfully reconfirmed to be positive for the presence of respective pathogens *N. meningitidis*, *S. pneumoniae* or *H. influenzae*,

using real-time PCR (Supplemental Table 4.4). Compared to these PCR results, the singleplex LEC-LAMP *N. meningitidis* assay demonstrated 100% diagnostic sensitivity and specificity by successfully detecting all *N. meningitidis* positive clinical samples and none of the *S. pneumoniae* or *H. influenzae* positive clinical samples (Supplemental Table 4.4). The positive controls reactions carried out in parallel were successfully detected with no detection observed in the NTC reactions.

#### 4.3.3 Singleplex LEC-LAMP *N. meningitidis* assay SNP identification with comparison to TEC-LAMP

Single-base specificity was successfully demonstrated in the singleplex LEC-LAMP *N. meningitidis* assay as only templates without SNPs, *N. meningitidis* genomic DNA (Figure 4.3A, blue) and the SNP0 template (Figure 4.3A, red), were successfully detected. All templates incorporating SNPs, SNP1-6, were not detected with the LEC-LAMP assay (Figure 4.3A, dashed red). The singleplex TEC-LAMP *N. meningitidis* assay successfully detected templates without SNPs, *N. meningitidis* genomic DNA (Figure 4.3B, blue) and the SNP0 template (Figure 4.3B, red). However, the TEC-LAMP assay did not demonstrate single-base specificity as it also detected the SNP incorporating template, SNPA (Figure 4.3B, dashed red). The no template control reactions performed successfully in both assays as no signal was observed (Figure 4.3, black).

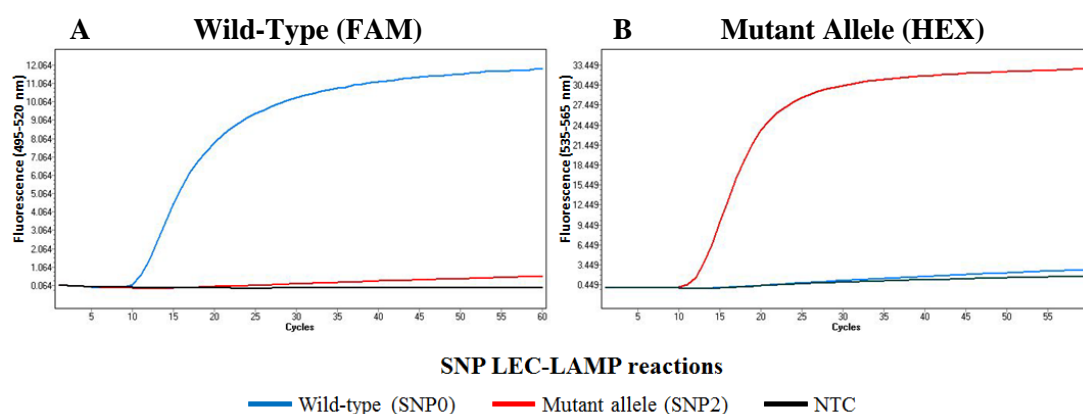


**Figure 4.3: Singleplex LEC-LAMP *N. meningitidis* assay SNP identification with comparison to TEC-LAMP.** The singleplex LEC-LAMP and TEC-LAMP *N. meningitidis* assays were separately challenged with templates without SNPs, *N. meningitidis* genomic DNA (blue) and SNP0 (red), at  $10^5$  copies. In parallel, the LEC-LAMP and TEC-LAMP

assays were also challenged with templates containing SNPs, SNP1-6 (A, dashed red) and SNPA (B, dashed red) respectively, at  $10^5$  copies. No template control (NTC) reactions were performed in parallel (black). Resulting amplification curves indicate LEC-LAMP single-base specificity as only templates without SNPs were detected. Single-base specificity in the TEC-LAMP assay was not observed as all templates, with or without SNPs, were detected. The NTC reactions performed successfully as no detection was observed.

#### 4.3.4 SNP LEC-LAMP *N. meningitidis* assay single-tube detection of either wild-type or mutant allele templates

The SNP LEC-LAMP *N. meningitidis* assay, incorporating FAM labelled wild-type LEC primer/probe and HEX labelled mutant SNP2 specific LEC primer/probe, successfully demonstrated differential detection of wild-type or mutant allele templates at  $10^5$  copies, in single-tube reactions (Figure 4.4). The SNP0 template, acting as the wild-type template, was successfully detected by the wild-type specific FAM labelled LEC primer/probe in the FAM detection channel (Figure 4.4A, blue), with no detection of this template observed in the HEX detection channel (Figure 4.4B, blue). The SNP2 template, acting as the mutant allele template, was successfully detected by the mutant allele SNP2 specific HEX labelled LEC primer/probe in the HEX detection channel (Figure 4.4B, red), with no detection of this template observed in the FAM detection channel (Figure 4.4A, red). The NTC reaction performed successfully as no signal was observed (Figure 4.4, black).

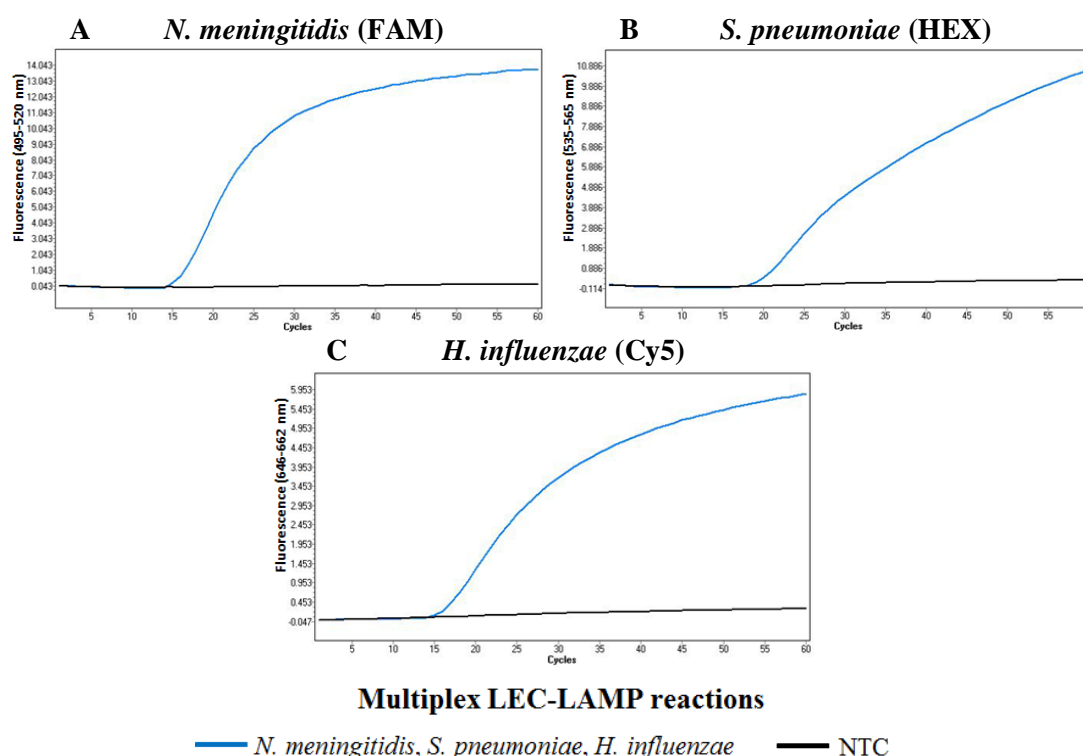


**Figure 4.4: SNP LEC-LAMP *N. meningitidis* assay single-tube detection of either wild-type or mutant allele templates.** The SNP LEC-LAMP *N. meningitidis* assay was separately challenged with the wild-type template SNP0 (blue), and the mutant allele template SNP2 (red), at  $10^5$  copies. A NTC reaction was performed in parallel (black). Resulting LAMP fluorescence signal was recorded in the LightCycler® 480 FAM (wild-type) and HEX (mutant allele) detection channels, with representative amplification curves for each reaction shown.

Successful LEC-LAMP detection of both templates was observed in respective detection channels only, with no unspecific detection of either template in non-corresponding channels observed. The NTC reaction performed successfully as no detection was observed.

#### 4.3.5 Multiplex LEC-LAMP *N. meningitidis*, *S. pneumoniae* and *H. influenzae* assay simultaneous multiple-target detection

Simultaneous multiple-target detection of all three bacterial templates at  $10^2$  genome copies, in a single reaction, was successfully demonstrated using the multiplex LEC-LAMP *N. meningitidis*, *S. pneumoniae* and *H. influenzae* assay (Figure 4.5, blue). Each target was detected in the appropriate respective fluorescence detection channel of the LightCycler® 480, *N. meningitidis* FAM (Figure 4.5A), *S. pneumoniae* HEX (Figure 4.5B) and *H. influenzae* Cy5 (Figure 4.5C). The NTC reaction performed successfully as no amplification was observed (Figure 4.5, black)



**Figure 4.5: Multiplex LEC-LAMP *N. meningitidis*, *S. pneumoniae* and *H. influenzae* assay simultaneous multiple-target detection.** The multiplex LEC-LAMP assay was challenged with three bacterial templates, *N. meningitidis* (A, blue), *S. pneumoniae* (B, blue) and *H. influenzae* (C, blue), at  $10^2$  genome copies in a single reaction. A NTC reaction was also performed in parallel (black). Resulting LAMP fluorescence signal was recorded in the LightCycler® 480 FAM (*N. meningitidis*), HEX (*S. pneumoniae*) and Cy5 (*H. influenzae*) detection channels with representative amplification curves for each reaction shown.

Successful simultaneous detection of all three bacterial templates, with no amplification in the NTC control reaction, was observed.

#### 4.4 Discussion

Loop-mediated isothermal amplification (LAMP) provides rapid, robust, sensitive and specific, user-friendly, infectious disease diagnostics technology for POC application in low-resourced disease-burdened areas. However, single nucleotide polymorphism (SNP) identification and multiplex pathogen detection is difficult to achieve using LAMP [88]. Nucleic acid diagnostics requires multiplex detection capabilities to facilitate simultaneous multiple-pathogen detection, reduced analysis time, sample conservation and incorporation of competitive [176] or non-competitive [88] internal control validation [52]. SNP identification capabilities in nucleic acid diagnostics enables effective differentiation of closely related pathogens, identification of point-mutations associated with antimicrobial resistances and more effective disease epidemiological surveillance/control [26, 173-175]. This study introduces LEC-LAMP technology for singleplex or multiplex pathogen detection with single-base specificity for flexible SNP identification. We demonstrated single-target detection and SNP identification using a singleplex LEC-LAMP assay, compared this to our previously reported TEC-LAMP technology, and evaluated in terms of analytical specificity, sensitivity and clinical application. Modified versions of this assay were subsequently used to demonstrate single-tube wild-type and mutant allele differentiation, and simultaneous multiple-pathogen detection.

The singleplex LEC-LAMP *N. meningitidis* assay demonstrated earlier detection and increased fluorescence production compared to TEC-LAMP (Figure 4.2), highlighting improved assay performance. This is possibly due to the significantly lower cleavage enzyme concentration in LEC-LAMP producing enhanced reaction kinetics, and the increased cleavage activity of endonuclease IV in LEC-LAMP, compared to *Tth* endonuclease IV in TEC-LAMP. In addition, the inner primers are essential to loop formation in LAMP and the TEC-LAMP 5'-end inner primer modifications could be effecting loop structure formation, whereas the LEC-LAMP inner primers are unmodified. The singleplex LEC-LAMP *N. meningitidis* assay was 100% analytically specific, as only the inclusivity panel reference strains were detected during specificity testing (Supplemental Table 4.1). The LOD of the singleplex LEC-LAMP *N.*

*meningitidis* assay was established to be 3.1 genome copies per reaction using Probit analysis (Supplemental Table 4.2). Typical LODs for previously reported *N. meningitidis* LAMP assays range from 6-10 genome copies per reaction [84, 148, 156]. Small scale clinical sample testing of the singleplex LEC-LAMP *N. meningitidis* assay, using DNA from *N. meningitidis*, *S. pneumoniae* and *H. influenzae* positive blood and CSF samples, demonstrated 100% diagnostic sensitivity and specificity as only the *N. meningitidis* samples were detected (Supplemental Table 4.4). All clinical samples were collected and processed by the IMSRL during routine diagnostic services in accordance with ethical review committee approved protocols. Samples were not analysed for human DNA in this study, and thus, the Ethics Committee of the National University of Ireland, Galway deemed that ethical approval for the evaluation of these samples was not required.

This study demonstrated that our previously reported TEC-LAMP assay does not enable single-base specificity as it amplified templates with and without SNPs located in the TEC primer/probe target region (Figure 4.3B). Presence of a SNP in the 5'-end of the TEC primer/probe target region could possibly inhibit binding during loop formation preventing abasic site dsDNA formation which in turn would inhibit cleavage and theoretically enable SNP identification. However, we hypothesise that the downstream *Bst* polymerisation, which dissociates the upstream loop structure, subsequently extends over the TEC primer/probe abasic site producing dsDNA which enables cleavage and fluorescence production regardless of the presence of a SNP. The LEC-LAMP technology reported in this study successfully demonstrated single-base specificity as none of the SNP containing templates tested, SNP1-6 (Table 4.2 and Supplemental Table 4.5), were detected (Figure 4.3A). We suggest that this single-base specificity in LEC-LAMP is possible for a number of reasons. Firstly, the downstream *Bst* polymerisation that prevents SNP identification in TEC-LAMP is not a factor in LEC-LAMP. Also, a combination of reaction temperature and the presence of a SNP inhibiting LEC primer/probe target hybridisation, prevents cleavage of the abasic site and enables the flexible SNP detection in LEC-LAMP. The demonstration of SNP identification in this study (Figure 4.3A) further indicates that nucleotide mismatches located within a specific 6 base region of the LEC primer/probe target (Table 4.2) prevents detection. SNPs located directly outside this 6 base range on the LEC primer/probe target were also tested but identified as positive with the

LightCycler® 480. However, the resulting amplification curves for these templates indicated low level detection which was easily differentiable from the SNP0 positive control reaction used. Based on these results, LEC-LAMP technology demonstrates flexible assay design for obtaining SNP identification, as targeted nucleotide mismatches can be located anywhere within this 6 base region (Table 4.2). To our knowledge, there is currently no other real-time multiplex isothermal nucleic acid amplification method that offers this type of flexible SNP identification. Further to this, utilising the loop primer for SNP identification enables even greater design flexibility, as once the core inner and outer primer sequences are confirmed, different loop primers can be designed to target various regions of the loop sequence, and thus, various SNPs that may be present. For convenience purposes, we demonstrated LEC-LAMP SNP identification in this study using synthetic 500 bp DNA gBlocks® Gene Fragments instead of genomic DNA. However, the resulting LEC-LAMP and TEC-LAMP amplification curves produced with these templates were very similar to resulting amplification curves for *N. meningitidis* genomic DNA (Figure 4.3).

Modifications were made to the singleplex LEC-LAMP *N. meningitidis* assay, including addition of a mutant LEC primer/probe (Table 4.1) to create the SNP LEC-LAMP assay, and addition of *S. pneumoniae* and *H. influenzae* oligonucleotides (Table 4.1) to create the multiplex LEC-LAMP assay. The SNP LEC-LAMP assay successfully demonstrated a single-tube reaction that can detect either wild-type or mutant allele templates (Figure 4.4). Other LAMP technologies reporting SNP detection capabilities require separate reactions to independently detect wild-type or mutant allele templates [89], increasing reagent and sample specimen costs. The multiplex LEC-LAMP assay successfully demonstrated the simultaneous detection of *N. meningitidis*, *S. pneumoniae* and *H. influenzae* (Figure 4.5) in a single reaction. This result highlights the robustness of LEC-LAMP in terms of tolerance to co-amplification inhibition which is necessary for multiplex nucleic acid diagnostics in the occurrence of pathogen co-infection [131]. The demonstration of multiplex LEC-LAMP detection also highlights the potential of this technology for incorporation of internal amplification controls, as with TEC-LAMP [88]. Multiplex LEC-LAMP was performed at the lower temperature of 65°C, compared to 67°C in singleplex LEC-LAMP, as this produced optimal detection of all three bacterial targets while maintaining efficient SNP identification capabilities. The combination of SNP LEC-

LAMP and multiplex LEC-LAMP capabilities highlights the potential for multiplex detection of closely positioned SNPs using a single core LAMP primer set. Previous PCR-based SNP detection technologies have been reported for the detection of SNPs clustered together in close proximity [177, 178]. Based on the multiplex capabilities of LEC-LAMP, the addition of further LEC primer/probes to the SNP LEC-LAMP assay, each targeting different SNPs and labelled with alternative fluorophores, could enable simultaneous detection of multiple closely located SNPs. There is also the option of modifying the reverse loop primer to target SNPs located in the opposing loop structure, expanding the application of LEC-LAMP.

Various LAMP technologies with SNP and point-mutation identification capabilities have been reported [87, 89, 169, 179-187], however, these methodologies have limitations compared to LEC-LAMP. Most reported methods employ allele-specific forward and reverse inner primers containing overlapping 5'-end mismatches to a specific SNP [89, 179-182, 184, 187], preventing hybridisation and amplification, and thus enabling differentiation between templates with and without this SNP. This strategy, however, has restrictive assay design as the entire LAMP primer set is based around a single nucleotide location. Also, this approach cannot guarantee complete SNP template amplification suppression [188], often leading to delayed SNP template detection and the occurrence of false positives or non-specific amplification [182]. Monitoring this methods is generally performed using intercalating dyes [179, 182], which can inhibit LAMP reactions [189-191], or real-time turbidimetry [181]. Both monitoring approaches, however, do not facilitate multiplex detection and require extensive optimisation to avoid non-specific amplification. This allele-specific design strategy could be applied to TEC-LAMP to enable SNP detection, however, the previously mentioned limitations would still apply and the flexible SNP detection of LEC-LAMP would not be possible. Yamanaka and colleagues recently reported SNP LAMP techniques using allele-specific oligonucleotide hybridisation (LAMP-ASO) and annealing selectivity of allele-specific inner primers with 3'-end or 5'-end mismatches (3'AS-LAMP and 5'AS-LAMP) [186]. However, LAMP-ASO is not a single-tube system requiring contamination prone post-amplification processing, and the AS-LAMP methods are prone to false-positive results. Carlos and colleagues report the use of gold nanoparticles to achieve SNP LAMP [185]. This method, however, is non-isothermal as template denaturing at 95°C is required, with laborious

contamination prone post-amplification processing. Lu and colleagues also report the use of gold nanoparticles to achieve SNP LAMP [169], however, this method again requires post-amplification analysis.

We have demonstrated in this study that the inner primers are not suitable for achieving LAMP SNP identification with the TEC primer/probe modifications (Figure 4.3B). Also, considering the outer primers do not target the unique double-looped LAMP template structure, we chose the loop primers for development of LEC-LAMP technology. A small number of LAMP technologies have reported real-time detection and SNP identification capabilities through utilisation of the loop primers. FLOS-LAMP demonstrates real-time LAMP detection using self-quenching and de-quenching fluorogenic loop probes [192]. However, this technology has not demonstrated multiplex target detection, does not enable SNP identification, has a relatively poor LOD of 500 genome copies, and possesses restrictive design limitations requiring guanine or cytosine flanked thymine residues for probe design. PNA-LNA LAMP has demonstrated SNP detection capabilities via an amplification blocking peptide nucleic acid (PNA) clamping probe that targets the loop regions in LAMP [193]. However, this method does not enable multiplex detection and requires post-amplification PCR or gel electrophoresis analysis. LAMP-FLP incorporates a fluorescently labelled loop primer (FLP), and quencher probe (QP), enabling SNP detection by measuring resulting peak temperatures of fluorescence resonance energy transfer (FRET) [183]. This method, however, requires increased oligonucleotide primer compared to standard LAMP, and is not fully isothermal as generation of a post-amplification annealing curve from 95°C to 35°C is required. Mohon and colleagues recently reported a SNP LAMP technique using a similar principle to the SNP overlap allele-specific inner primer methods [87]. This method, however, utilises overlap between the loop and inner primer, and is subject to the same previously mentioned limitations of restrictive assay design and monitoring using intercalating dyes or turbidity.

LEC-LAMP incorporates all of the previously reported properties of TEC-LAMP technology, in terms of assay specificity, sensitivity and improved multiplex target detection over competing methods. However, LEC-LAMP technology further improves on TEC-LAMP in terms of reaction time-to-positivity and fluorescence production, as well as now incorporating flexible SNP identification capabilities.

Additionally, LEC-LAMP requires one fifteenth of the cleavage enzyme concentration, and half of the oligonucleotide probe concentration, required by TEC-LAMP. The LEC primer/probe also uses alternate fluorophore and quencher positioning to TEC-LAMP, this has no impact on assay performance but further reduces assay cost in terms of oligonucleotide synthesis. Further reduced assay costs can be achieved by lowering the LEC-LAMP primer and probe concentrations by half, maintaining comparable detection times and fluorescence production with single-digit genome copy LOD. LEC-LAMP is the first report of a single-tube, real-time, multiplex LAMP method with flexible SNP identification capabilities, providing state-of-the-art transferable isothermal nucleic acid amplification technology for POC infectious disease diagnostics.

#### 4.5 Supplementary data

<b>Table 4.1: Singleplex LEC-LAMP <i>Neisseria meningitidis</i> assay specificity panel.</b>		
Organism	Strain	LEC-LAMP Result
<b>Inclusivity Panel</b>		
<i>N. meningitidis</i> reference strains		
<i>N. meningitidis</i> (A, type strain)	NCTC 10025	+
<i>N. meningitidis</i> (A)	DSM 10036	+
<i>N. meningitidis</i> (A)	NCTC 3372	+
<i>N. meningitidis</i> (A)	NCTC 3375	+
<i>N. meningitidis</i> (B)	ATCC 13090	+
<i>N. meningitidis</i> (C)	ATCC 13102	+
<i>N. meningitidis</i> (C)	DSM 15464	+
<i>N. meningitidis</i> (W)	NCTC 11203	+
<i>N. meningitidis</i> (X)	NCTC 10790	+
<i>N. meningitidis</i> (Y)	NCTC 10791	+
<b>Exclusivity Panel</b>		
<i>Neisseria</i> reference strains and closely related non- <i>Neisseria</i> reference strains		
<i>N. animalis</i>	DSM 23392	-
<i>N. animaloris</i>	DSM 21642	-
<i>N. bacilliformis</i>	DSM 23338	-
<i>N. canis</i>	DSM 18000	-
<i>N. caviae</i>	DSM 23336	-
<i>N. cuniculi</i>	DSM 21768	-
<i>N. dentiae</i>	DSM 19151	-
<i>N. elongata</i> subsp. <i>elongata</i>	DSM 17712	-
<i>N. elongata</i> subsp. <i>glycolytica</i>	DSM 23337	-
<i>N. elongata</i> subsp. <i>nitroreducens</i>	DSM 17632	-
<i>N. flavescens</i>	DSM 17633	-
<i>N. gonorrhoeae</i>	ATCC 19424	-
<i>N. gonorrhoeae</i>	DSM 9188	-
<i>N. gonorrhoeae</i>	DSM 9189	-

<i>N. lactamica</i>	ATCC 23970	-
<i>N. lactamica</i>	DSM 4691	-
<i>N. macacae</i>	DSM 19175	-
<i>N. mucosa</i>	DSM 17611	-
<i>N. ovis</i>	DSM 18075	-
<i>N. perflava</i>	DSM 18009	-
<i>N. polysaccharea</i>	DSM 22809	-
<i>N. shayeganii</i>	DSM 22246	-
<i>N. sicca</i>	DSM 17713	-
<i>N. subflava</i>	DSM 17610	-
<i>N. wadsworthii</i>	DSM 22247	-
<i>N. weaveri</i>	DSM 17688	-
<i>N. zoodegmatis</i>	DSM 21483	-
<i>N. zoodegmatis</i>	DSM 21643	-
<i>H. influenzae</i>	DSM 4690	-
<i>H. parainfluenzae</i>	DSM 8978	-
<i>H. haemolyticus</i>	CCUG 15312	-
<i>H. sommus</i>	CCUG 12839	-
<i>S. pneumoniae</i>	DSM 20566	-
<i>S. pseudopneumoniae</i>	DSM 18670	-
<i>S. agalactiae</i>	BCCM 15081	-
<i>S. mitis</i>	DSM 12643	-
<i>K. pneumoniae</i>	DSM 30104	-
<i>P. aeruginosa</i>	DSM 50071	-
<i>E. coli</i>	DSM 30083	-
<i>E. faecalis</i>	DSM 20371	-
<i>S. aureus</i>	DSM 346	-

NCTC, National Collection of Type Cultures; DSM, Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures; ATCC, American Type Culture Collection; CCUG, Culture Collection, University of Göteborg, Sweden; BCCM, Belgian Coordinated Collections of Microorganisms; +, positive; -, negative.

**Table 4.2: Singleplex LEC-LAMP *N. meningitidis* assay limit of detection (LOD) Probit analysis.**

Genome copy concentration tested	Replicates tested / Replicates detected
32	6 / 6
16	6 / 6
8	6 / 6
4	6 / 6
2	6 / 4
1	6 / 1
<b>Genome copy LOD per reaction (95% probability)</b>	<b>3.1</b>

**Table 4.3: PCR oligonucleotides.**

Type	Sequence (5'-3')
<i>N. meningitidis</i>	
Forward	CGACATGTTCTGAACGTAATCTCC
Probe	(FAM)TATCGGGCAAAGCCAAATGCGAAG(BHQ1)
Reverse	ATTTCTGGTGGCGCGTTT
<i>S. pneumoniae</i>	
Forward	CTCGTAAGCGTAAACTCCTTG
Probe	(FAM)ACGCATGAAATCCATCGGATCAGTT(BHQ1)
Reverse	CATACTCAAGACGCTGAGGA
<i>H. influenzae</i>	

Forward	GGTACGCACYACGGACAATATG
Probe	(FAM)AGCTCTTGGTTGCTCTCAATGGCA(BHQ1)
Reverse	CCTGATTTAGCYGCTCGATAACA
FAM, 6-carboxyfluorescein fluorophore; BHQ1, black hole quencher	

**Table 4.4: Singleplex LEC-LAMP *N. meningitidis* assay clinical sample testing using *N. meningitidis*, *S. pneumoniae* and *H. influenzae* PCR-positive DNA extracts from blood and CSF specimens of confirmed bacterial meningitis cases.**

<b><i>N. meningitidis</i></b>			
<b>Clinical Samples</b>			
Sample No.	Clinical Specimen	<i>N. meningitidis</i> PCR (Ct Value)	<i>N. meningitidis</i> LEC-LAMP (Ct Value)
1	BLD	36.97	24.30
2	BLD	30.82	15.06
3	BLD	31.46	14.49
4	BLD	30.02	15.47
5	BLD	30.05	15.05
6	BLD	34.17	16.26
7	CSF	33.55	17.23
8	BLD	36.11	37.13
9	CSF	30.21	13.28
10	BLD	33.06	16.82
11	CSF	30.32	15.27
12	BLD	33.17	17.62
13	BLD	36.37	21.37
14	BLD	33.47	17.73
15	BLD	23.82	12.01
16	BLD	36.72	17.86
17	BLD	33.91	16.00
18	CSF	36.46	23.05
19	BLD	29.88	14.62
20	BLD	34.83	18.28
21	BLD	33.89	17.37
22	BLD	36.06	12.23
23	CSF	34.02	16.84
24	CSF	23.98	13.29
25	CSF	32.79	18.20
26	CSF	23.04	13.53
27	CSF	33.31	18.49
28	CSF	22.39	13.27
29	CSF	27.29	13.65
30	CSF	26.53	12.60
31	CSF	22.98	13.75
32	CSF	30.30	14.52
33	CSF	26.93	12.11
<b><i>S. pneumoniae</i></b>			
<b>Clinical Samples</b>			
Sample No.	Clinical Specimen	<i>S. pneumoniae</i> PCR (Ct Value)	<i>N. meningitidis</i> LEC-LAMP (Ct Value)
34	BLD	31.61	-
35	CSF	24.13	-
36	BLD	28.01	-
37	CSF	34.64	-
38	CSF	26.33	-
39	BLD	28.59	-
40	CSF	30.47	-
41	CSF	33.22	-

42	CSF	31.99	-
43	CSF	25.32	-
44	BLD	31.84	-
45	BLD	26.87	-
46	CSF	29.31	-
47	BLD	27.05	-
48	BLD	32.61	-
49	CSF	25.60	-
50	CSF	23.18	-
51	CSF	28.83	-
52	CSF	23.61	-
53	CSF	26.34	-
54	CSF	31.06	-
55	CSF	28.81	-
<b><i>H. influenzae</i></b> <b>Clinical Samples</b>			
Sample No.	Clinical Specimen	<i>H. influenzae</i> PCR (Ct Value)	<i>N. meningitidis</i> LEC-LAMP (Ct Value)
56	BLD	38.50	-
57	CSF	37.26	-
58	BLD	37.48	-
59	CSF	36.54	-
60	CSF	32.24	-
61	CSF	28.65	-
62	CSF	27.95	-
63	BLD	35.50	-
64	BLD	38.40	-
65	BLD	37.73	-
66	BLD	34.44	-
67	BLD	38.61	-
68	BLD	31.18	-
69	BLD	38.45	-
70	BLD	37.13	-
71	CSF	35.73	-
72	CSF	30.99	-

BLD, blood; CSF, cerebrospinal fluid; -, negative.

**Table 4.5: *N. meningitidis* 500 bp DNA gBlocks® Gene Fragments with and without SNPs.**

<b>SNP0 (without SNP)</b> AGCTGTAATACCACATCACCGCCAGAAAGATAAACGAAAACGCCAAATCCAACACCGAAGTCAGCGCCTGACCGGTC AAGAAATTGCGAATCTGCTCCAATTCCCGCACCCGAGCCACCGTATCACCCTCGTCTGTGCTCGAAATAGGATAA AGGCAGGGAAAGCAGATGCCGGAACAAACGCGCGCCCAATTCACATCAATACGTGAAGTCGTATGTGCAAACAGAT ACGTCCGCAAACCGCCCAACACAATCTCAAACAGCGACACCACCAACAAAGCCACCGACACCACATCCAAAGTAGAG AATCCCCGATGTACCAGCACCTTGTCATCACCCTTGAAAAACAGAGGCGTAATCAGCGCAAACAGCTGCAACAC CACCAGACACCACCAATACTTCAAAAAACAACCGGCGGTATTTGATTACCGCCGGAATAAACAGGTAAAGTCAAACCT TTGCCAAACTGCCCAATACCGAAGCGCGGGAAGCAACC
<b>SNP1 (with SNP)</b> AGCTGTAATACCACATCACCGCCAGAAAGATAAACGAAAACGCCAAATCCAACACCGAAGTCAGCGCCTGACCGGTC AAGAAATTGCGAATCTGCTCCAATTCCCGCACCCGAGCCACCGTATCACCCTCGTCTGTGCTCGAAATAGGATAA AGGCAGGGAAAGCAGATGCCGGAACAAACGCGCGCCCAATTCACATCAATACGTGAAGTCGTATGTGCAAACAGAT ACGTCCGCAAACCGCCCAACACAATCGCAAACAGCGACACCACCAACAAAGCCACCGACACCACATCCAAAGTAGAG AATCCCCGATGTACCAGCACCTTGTCATCACCCTTGAAAAACAGAGGCGTAATCAGCGCAAACAGCTGCAACAC CACCAGACACCACCAATACTTCAAAAAACAACCGGCGGTATTTGATTACCGCCGGAATAAACAGGTAAAGTCAAACCT TTGCCAAACTGCCCAATACCGAAGCGCGGGAAGCAACC
<b>SNP2 (with SNP)</b> AGCTGTAATACCACATCACCGCCAGAAAGATAAACGAAAACGCCAAATCCAACACCGAAGTCAGCGCCTGACCGGTC AAGAAATTGCGAATCTGCTCCAATTCCCGCACCCGAGCCACCGTATCACCCTCGTCTGTGCTCGAAATAGGATAA AGGCAGGGAAAGCAGATGCCGGAACAAACGCGCGCCCAATTCACATCAATACGTGAAGTCGTATGTGCAAACAGAT ACGTCCGCAAACCGCCCAACACAAGCTCAAACAGCGACACCACCAACAAAGCCACCGACACCACATCCAAAGTAGAG AATCCCCGATGTACCAGCACCTTGTCATCACCCTTGAAAAACAGAGGCGTAATCAGCGCAAACAGCTGCAACAC

CACCGACACCACCAATACTTCAAAAAACAACCGGGGGTATTTGATTACCGCCGGAATAAACAGGTAAAGTCAAAC TTGCCAAACTGCCCAATACCGAAGCGGGGAAGCAACC
<b>SNP3 (with SNP)</b> AGCTGTAATACCACATCACCGCCAGAAAGATAAACGAAAAAGCCAAATCCAACACCGAAGTCAGCGCCTGACCGGTC AAGAAATTGCGAATCTGCTCCAATTCCCGCACCCGAGCCACCGTATCACCCACTCGTCTGTGCTCGAAATAGGATAA AGGCAGGGAAAGCAGATGCCGGAACAAACGCGCGCCCAATTCACATCAATACGTGAAGTCGTATGTGCAACAGAT ACGTCCGCAAACCGCCCAACACACTCTCAAACAGCGACACCACCAACAAAGCCACCGACACCACATCCAAAGTAGAG AATCCCCGATGTACCAGCACCTTGTCATCACCACTTGAAAAACAGAGGCGTAATCAGCGCAAACAGCTGCAACAC CACCGACACCACCAATACTTCAAAAAACAACCGGGGGTATTTGATTACCGCCGGAATAAACAGGTAAAGTCAAAC TTGCCAAACTGCCCAATACCGAAGCGGGGAAGCAACC
<b>SNP4 (with SNP)</b> AGCTGTAATACCACATCACCGCCAGAAAGATAAACGAAAAAGCCAAATCCAACACCGAAGTCAGCGCCTGACCGGTC AAGAAATTGCGAATCTGCTCCAATTCCCGCACCCGAGCCACCGTATCACCCACTCGTCTGTGCTCGAAATAGGATAA AGGCAGGGAAAGCAGATGCCGGAACAAACGCGCGCCCAATTCACATCAATACGTGAAGTCGTATGTGCAACAGAT ACGTCCGCAAACCGCCCAACACACTCTCAAACAGCGACACCACCAACAAAGCCACCGACACCACATCCAAAGTAGAG AATCCCCGATGTACCAGCACCTTGTCATCACCACTTGAAAAACAGAGGCGTAATCAGCGCAAACAGCTGCAACAC CACCGACACCACCAATACTTCAAAAAACAACCGGGGGTATTTGATTACCGCCGGAATAAACAGGTAAAGTCAAAC TTGCCAAACTGCCCAATACCGAAGCGGGGAAGCAACC
<b>SNP5 (with SNP)</b> AGCTGTAATACCACATCACCGCCAGAAAGATAAACGAAAAAGCCAAATCCAACACCGAAGTCAGCGCCTGACCGGTC AAGAAATTGCGAATCTGCTCCAATTCCCGCACCCGAGCCACCGTATCACCCACTCGTCTGTGCTCGAAATAGGATAA AGGCAGGGAAAGCAGATGCCGGAACAAACGCGCGCCCAATTCACATCAATACGTGAAGTCGTATGTGCAACAGAT ACGTCCGCAAACCGCCCAACAAATCTCAAACAGCGACACCACCAACAAAGCCACCGACACCACATCCAAAGTAGAG AATCCCCGATGTACCAGCACCTTGTCATCACCACTTGAAAAACAGAGGCGTAATCAGCGCAAACAGCTGCAACAC CACCGACACCACCAATACTTCAAAAAACAACCGGGGGTATTTGATTACCGCCGGAATAAACAGGTAAAGTCAAAC TTGCCAAACTGCCCAATACCGAAGCGGGGAAGCAACC
<b>SNP6 (with SNP)</b> AGCTGTAATACCACATCACCGCCAGAAAGATAAACGAAAAAGCCAAATCCAACACCGAAGTCAGCGCCTGACCGGTC AAGAAATTGCGAATCTGCTCCAATTCCCGCACCCGAGCCACCGTATCACCCACTCGTCTGTGCTCGAAATAGGATAA AGGCAGGGAAAGCAGATGCCGGAACAAACGCGCGCCCAATTCACATCAATACGTGAAGTCGTATGTGCAACAGAT ACGTCCGCAAACCGCCCAACCAATCTCAAACAGCGACACCACCAACAAAGCCACCGACACCACATCCAAAGTAGAG AATCCCCGATGTACCAGCACCTTGTCATCACCACTTGAAAAACAGAGGCGTAATCAGCGCAAACAGCTGCAACAC CACCGACACCACCAATACTTCAAAAAACAACCGGGGGTATTTGATTACCGCCGGAATAAACAGGTAAAGTCAAAC TTGCCAAACTGCCCAATACCGAAGCGGGGAAGCAACC
<b>SNPA (with SNP)</b> AGCTGTAATACCACATCACCGCCAGAAAGATAAACGAAAAAGCCAAATCCAACACCGAAGTCAGCGCCTGACCGGTC AAGAAATTGCGAATCTGCTCCAATTCCCGCACCCGAGCCACCGTATCACCCACTCGTCTGTGCTCGAAATAGGATAA AGGCAGGGAAAGCAGATGCCGGAACAAACGCGCGCCCAATTCACATCAATACGTGAAGTCGTATGTGCAACAGAT ACGTCCGCAAACCGCCCAACACAATCTCAAACAGCGACACCACCAACAAAGCCACTACACCACATCCAAAGTAGAG AATCCCCGATGTACCAGCACCTTGTCATCACCACTTGAAAAACAGAGGCGTAATCAGCGCAAACAGCTGCAACAC CACCGACACCACCAATACTTCAAAAAACAACCGGGGGTATTTGATTACCGCCGGAATAAACAGGTAAAGTCAAAC TTGCCAAACTGCCCAATACCGAAGCGGGGAAGCAACC
Highlight, SNP mismatch.

**Chapter 5**  
**Conclusions & Future Research**

## 5.1 Conclusions

The focus of this thesis was to advance the state-of-the-art in multiplex isothermal nucleic acid amplification technologies for the detection of *S. pneumoniae*, *N. meningitidis* and *H. influenzae*, major bacterial meningitis pathogens. Recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP) technologies were the single-temperature diagnostic methods utilised in this research project. The initial aim was to gain experience in the design and development of RPA assays incorporating unique real-time probing technologies, and to apply this experience in the development of novel real-time probing methods for LAMP. Development of the RPA and LAMP assays in Chapters 2, 3 and 4 of this study began with determining the diagnostic targets to be used for the identification of each pathogen. The RPA assays detailed in Chapter 2 identified *S. pneumoniae* and *H. influenzae* by targeting the elongation factor 4 (EF4)/*lepA* gene and L-fuculose kinase/*fucK* gene, respectively. Alternative *S. pneumoniae* and *H. influenzae* targets were used for the LAMP technologies in Chapters 3 and 4, the *SPNA45\_01710* and *pstA* genes, respectively. The *pstA* gene was recently highlighted as an effective *H. influenzae* biomarker and we identified the novel *SPNA45\_01710* gene biomarker using whole genome sequence alignment analysis. For the identification of *N. meningitidis* in Chapters 2, 3 and 4 we also identified the novel *NMO\_1242* gene biomarker using whole genome sequence alignment analysis. All assays were evaluated using extensive inclusivity and exclusivity panels of bacterial reference strains and clinical samples from confirmed cases of bacterial meningitis infection, provided by the Irish Meningitis and Sepsis Reference Laboratory (IMSRL), Dublin. All clinical samples were collected and processed during routine diagnostic services in accordance with ethical review committee approved protocols. Samples were not analysed for human DNA in this project, and thus, the Ethics Committee of the National University of Ireland, Galway deemed that ethical approval for evaluation of these samples was not required.

Chapter 2 of this thesis detailed the development and evaluation of three internally controlled duplex RPA assays for the detection of *S. pneumoniae*, *N. meningitidis* and *H. influenzae*. Singleplex RPA assays for each target were initially designed and evaluated. Each assay demonstrated 100% specificity. This result confirmed the utility of the novel *NMO\_1242* gene as an effective diagnostic target for *N. meningitidis*.

Also, as the leading pathogenic *N. meningitidis* and *H. influenzae* serotypes/serogroups were tested in this study, and the *S. pneumoniae lepA* diagnostic target is highly conserved in all known bacteria, the effective specificity of these assays was highlighted. The sensitivities of the *S. pneumoniae*, *N. meningitidis* and *H. influenzae* singleplex RPA assays were comparable to PCR at 4.1, 8.5 and 3.9 genome copies per reaction, respectively. Incorporation of internal amplification control (IAC) templates and corresponding RPA exo probes into the singleplex RPA assays was used to develop the duplex RPA assays in this study. Optimisation of these assays was required to determine the optimal IAC template concentrations that enabled both efficient IAC detection whilst maintaining efficient bacterial target detection. The robustness of these duplex RPA assays was also highlighted by successful demonstration of bacterial target detection in the presence of high concentrations of background DNA, with simultaneous IAC detection. Clinical sample testing of all three duplex RPA assays was performed using 64 archived PCR-positive DNA extracts from confirmed cases of pneumococcal, meningococcal and Haemophilus infection. Diagnostic specificity was 100% for each assay and diagnostic sensitivity was 100%, 85.7% and 100% for the *S. pneumoniae*, *N. meningitidis* and *H. influenzae* assays, respectively, producing significantly earlier detection times compared to PCR. All duplex RPA reactions in this study were successfully validated by the detection of the IAC template in all events of no bacterial target detection. The RPA assays detailed in this study were the first report and clinical evaluation of internally controlled duplex RPA assays for the detection of these major bacterial meningitis pathogens.

Chapter 3 of this thesis introduced our novel real-time, internally controlled, multiplex *Tth* endonuclease cleavage loop-mediated isothermal amplification (TEC-LAMP) assay for the detection of *S. pneumoniae*, *N. meningitidis* and *H. influenzae*. In this study we evaluated the analytical specificity, sensitivity and clinical performance of TEC-LAMP using in-house bacterial reference strains and clinical samples provided by the IMSRL. Complete analytical specificity of the TEC-LAMP assay was demonstrated. This confirmed the utility of our novel *SPNA45\_01710* gene as an effective biomarker for *S. pneumoniae*. The TEC-LAMP limit of detection (LOD) for *S. pneumoniae*, *N. meningitidis* and *H. influenzae*, in the presence of the IAC, was 39.5, 17.3 and 26.5 genome copies per reaction, respectively. These LODs compared favourably to previously reported multiplex PCR and singleplex LAMP assays for

these pathogens. TEC-LAMP clinical performance was assessed using 65 clinical sample DNA extracts that were PCR-positive for either *S. pneumoniae*, *N. meningitidis* or *H. influenzae*. Diagnostic sensitivity and specificity of the TEC-LAMP assay was established to be 92.3% and 100%, respectively, with positive results detected at an approximate average time-to-positivity of 20 min per sample, significantly faster than the IMSRL PCR results. TEC-LAMP detection of the IAC template in all events of no bacterial target detection successfully validated test results in this study. The demonstration of the internally controlled simultaneous multiplex detection of all three bacterial targets in this study highlighted the diagnostic utility of TEC-LAMP, especially in cases of co-infection. This study extensively highlighted the various assay performance advantages that TEC-LAMP has over conventional LAMP monitoring methodologies as well as more recent novel multiplex LAMP technologies, such as DARQ-LAMP, QUASR-LAMP and MERT-LAMP. TEC-LAMP is the first report of a single-tube internally controlled multiplex LAMP assay with clinically relevant analytical sensitivity, and the first clinical evaluation of such technology applied to the detection of these leading bacterial meningitis pathogens. This was also the first report of *Tth* endonuclease IV incorporation into nucleic acid amplification diagnostic technology.

Chapter 4 of this thesis detailed the introduction of our novel loop-primer endonuclease cleavage loop-mediated isothermal amplification (LEC-LAMP) technology, a further advance on state-of-the-art LAMP monitoring. LEC-LAMP enables singleplex or multiplex pathogen detection with flexible single-base specificity for the effective identification of single nucleotide polymorphisms (SNPs). As with the previous studies in Chapters 2 and 3, bacterial meningitis pathogens *N. meningitidis*, *S. pneumoniae* and *H. influenzae* were used to exemplify the diagnostic properties of LEC-LAMP. The SNP detection capabilities of this technology were demonstrated using synthetic templates containing specific single mismatch nucleotides, and a synthetic template without any mismatches as a control comparison. Single-target detection and SNP identification was demonstrated using a singleplex LEC-LAMP *N. meningitidis* assay. This was compared to our previous TEC-LAMP technology and further evaluated in terms of analytical specificity, sensitivity and clinical application. The singleplex LEC-LAMP *N. meningitidis* assay demonstrated improved assay performance compared to TEC-LAMP, with earlier detection times

and increased fluorescence production. The singleplex LEC-LAMP *N. meningitidis* assay was 100% specific with an LOD of 3.1 genome copies per reaction, which compares very favourably to other reported *N. meningitidis* LAMP assays. Clinical evaluation of the singleplex LEC-LAMP *N. meningitidis* assay, using DNA from *N. meningitidis*, *S. pneumoniae* and *H. influenzae* positive blood and CSF samples, demonstrated 100% diagnostic sensitivity and specificity. This study highlighted that our previous TEC-LAMP technology did not enable SNP identification as it detected templates both with and without SNPs. LEC-LAMP, however, successfully demonstrated efficient SNP identification capabilities as only templates without any SNPs were detected, while all templates with SNPs were not detected. Additionally, LEC-LAMP demonstrated flexible SNP identification capabilities as nucleotide mismatches located anywhere in a specific 6 base region enabled differentiation. Currently, there is no other real-time multiplex isothermal nucleic acid amplification method that offers this type of flexible SNP identification. Modifications were made to the singleplex LEC-LAMP *N. meningitidis* assay, including: addition of a mutant template specific probe to create the SNP LEC-LAMP assay; and addition of *S. pneumoniae* and *H. influenzae* oligonucleotides to create a multiplex LEC-LAMP assay. The SNP LEC-LAMP assay successfully demonstrated single-tube detection of either wild-type or mutant allele templates, and the multiplex LEC-LAMP assay successfully demonstrated simultaneous detection of *N. meningitidis*, *S. pneumoniae* and *H. influenzae* in a single reaction. This study extensively highlighted the advantages of LEC-LAMP over competing LAMP technologies in terms of target detection and SNP identification. LEC-LAMP incorporates all of the previously reported properties of TEC-LAMP, with now the added improvements in terms of reaction time-to-positivity and fluorescence production, as well as the incorporation flexible SNP identification capabilities. Additionally, LEC-LAMP requires one fifteenth of the cleavage enzyme concentration, and half of the oligonucleotide probe concentration, required by TEC-LAMP. LEC-LAMP is the first report of a single-tube, real-time, multiplex LAMP method with flexible SNP identification capabilities.

## 5.2 Future research

Various aspects of the novel TEC-LAMP and LEC-LAMP diagnostic technologies described in this thesis will be investigated for possible improvements to assay performance and application. Initially, both technologies will be modified to detect RNA templates, in combination with DNA templates. Targeting RNA instead of DNA in molecular diagnostics is often required to indicate the presence of viable infectious agents [60, 194, 195]. Also, diagnostic assays targeting RNA will theoretically enable improved sensitivity compared to targeting DNA, as RNA transcript copies are typically present at greater copy numbers than DNA in biological cells [196]. RNA detection using these LAMP technologies will be achieved by replacing the current *Bst* DNA polymerase with a newly developed strand-displacing DNA polymerase that also incorporates enhanced reverse transcriptase activity, *Bst* 3.0 DNA polymerase (New England Biolabs, UK). Following assay optimisation, this modification will enable the real-time, isothermal, single-base specific, multiplex LAMP detection of RNA templates in a single-tube reaction. Addition of a separate reverse transcriptase enzyme into the existing LAMP reactions, in combination with the *Bst* 2.0 DNA polymerase, could also achieve this result. However, in addition to the endonuclease cleavage enzymes, a two-enzyme system compared to a three-enzyme system is preferable in terms of assays cost and possible improved reaction kinetics due to less assay components.

Currently, both the TEC-LAMP and LEC-LAMP technologies utilise only one oligonucleotide primer direction for probe modifications, the forward inner primer in TEC-LAMP and the forward loop primer in LEC-LAMP. Each LAMP technology will be modified to incorporate respective primer/probe modifications on both the forward and reverse primer components, followed by assay performance evaluation. This modification could lead to improved assay performances in terms of increased fluorescence production and earlier detection times. Additionally, this approach of modifying both the forward and reverse primer sets will be applied to the LEC-LAMP technology in an effort to expand SNP identification capabilities. Currently, LEC-LAMP can only differentiate between closely located SNPs positioned in the forward loop primer target region. In the event of needing to identify, or differentiate between, SNPs located further apart, an additional LEC primer/probe targeting the reverse loop primer region could possibly achieve this.

An alternative internal amplification control (IAC) system will be designed and incorporated into the TEC-LAMP assay, followed by performance evaluation and comparison with the current assay. Typical IAC incorporation into nucleic acid amplification diagnostics for the purposes of assay validation uses either a non-competitive or competitive approach [52]. The current TEC-LAMP assay uses a non-competitive IAC approach with two oligonucleotide primer sets, one to detect the target template and the other to detect the IAC template. The competitive IAC approach uses one set of oligonucleotide primers to detect both the target template and IAC template. In the competitive IAC approach, the IAC template is usually embedded into a vector system, such as plasmid DNA. The oligonucleotide primers are designed to amplify both the target and IAC templates, however, differentiation between both targets is achieved using different oligonucleotide probes to target each individual template. Altering the TEC-LAMP IAC system to a competitive IAC would remove the need for an individual IAC primer set and significantly reducing the overall oligonucleotide composition of the TEC-LAMP assay, possibly improving reaction kinetics.

The future aim for the novel isothermal amplification technologies described in this thesis is the incorporation with point-of-care (POC) instrumentation for near-patient infectious disease diagnostic application. Various recently published studies have demonstrated the successful combination of RPA and LAMP with miniaturised, portable, easy-to-use POC devices [68-72, 150-154]. RPA is performed at a low close to body temperature range of 40°C, requiring minimal energy input that simplifies integration with POC technologies. TEC-LAMP and LEC-LAMP require a higher 67°C heat source, creating an increased challenge for incorporation with low-cost POC technologies, thus, investigation into lowering these reaction temperatures will be carried out. The *Bst* DNA polymerase used in these LAMP methods requires a 55-65°C temperature range for efficient polymerisation [90], and is not compatible with a lower 40°C reaction temperature. However, phi29 DNA polymerase from *Bacillus subtilis* phage phi29, possesses effective strand displacement amplification properties at 30-40°C temperatures [197]. This DNA polymerase has also been extensively used in other lower temperature isothermal amplification technologies, such as multiple displacement amplification (MDA) [198], providing a possible alternative strand displacement polymerase enzyme for low temperature LAMP reactions. The TEC-

LAMP reaction temperature cannot be lowered due the required 60-65°C temperature range needed by the *Tth* endonuclease IV enzyme. However, the endonuclease IV restriction enzyme used in LEC-LAMP maintains activity at the lower temperature range of 37°C [199], thus, in combination with the alternative phi29 DNA polymerase, the reaction temperature of LEC-LAMP could possibly be lowered. Lowering the LEC-LAMP reaction temperature would however inhibit the heat-mediated DNA template dissociation needed to enable primer hybridisation. LEC-LAMP relies solely on reaction temperature and strand-displacement polymerase activity to destabilise the template DNA, and unlike most reported LAMP methods, does not utilise template destabilising agents like betaine. Thus, incorporation of DNA destabilising reagents such as betaine, or proline, could possibly overcome this inhibition of heat-mediated template dissociation at a lower reaction temperature [200].

**Chapter 6**  
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