Loop mediated isothermal amplification to detect Respiratory Syncytial Virus in respiratory specimens.

by

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Declaration:

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Abstract:

Background: Respiratory Syncytial Virus (RSV) is the leading cause of severe lower respiratory tract infection in infants and children worldwide. Early diagnosis of RSV infection is associated with shorter periods of hospitalisation and decreased mortality. Current point of care (PoC) tests for RSV is less sensitive than molecular methods. Reverse transcription loop-mediated isothermal amplification (RT-LAMP), is a novel method of nucleic acid detection which allows for rapid, robust amplification, and visual detection of infectious agents.

Aim: The objective of this study was to develop a novel, rapid, and sensitive multiplex RSV RT-LAMP assay for PoC diagnosis of RSV A and B.

Methods: Preparation of a quantitative RSV standard for assay optimisation was done using a rapid hypotonic burst recovery method of infective virus during sub-passaging, and a shell vial fluorescent focus assay for titration of culture-derived viral stock. We designed a single set of eight primers targeting the large polymerase gene of both RSV A and B, and developed a novel single-step multiplex RSV RT-LAMP assay, using an in-house reaction mix and the Rotor-Gene Q real-time thermocycler (Qiagen, Hilden, Germany). The metal ion indicator hydroxy naphtol blue (HNB) was added to the multiplex RSV RT-LAMP assay for visual detection of RSV.

Results: The final optimised multiplex RSV RT-LAMP assay had an analytical detection sensitivity of <10 focus forming units (FFU) per reaction for both RSV A and B, with a mean time to positivity of 21.85 minutes (95% CI 19.2-24.5 minutes), compared to 90-120 minutes for conventional PCR. Evaluated against the Seeplex RV15 multiplex PCR (Seegene, Seoul, Korea) by testing 44 (22 RSV A/22 RSV B) nasopharyngeal specimens, the multiplex RSV RT-LAMP assay had a sensitivity of 100%, and a specificity of 100% when screened against nine common respiratory viruses. Visual detection of RSV using HNB as colorimetric reagent was equivalent to the analytical sensitivity (10 FFU/reaction) and specificity (100%) of the multiplex RSV RT-LAMP assay.

Conclusion: Compared with conventional PCR, our novel single-step multiplex RSV RT-LAMP assay had excellent sensitivity, specificity, and when combined with HNB dye could provide accurate visual diagnosis within 1 hour. We envisage that this multiplex RSV RT-LAMP assay will be used for rapid and sensitive RSV detection at the PoC.

Opsomming:

Agtergrond: Respiratoriese Syncytial Virus (RSV) is die hoof oorsaak van erge laer lugweginfeksie in babas en kinders wêreldwyd. Vroeë diagnose van RSV infeksie word geassosieer met korter periodes van hospitalisasie en verlaagde mortaliteit. Huidige punt van sorg (PoC) toetse vir RSV is minder sensitief as molekulêre metodes. Omgekeerde transkripsie lus-gemedieerde isotermiese amplifisering (RT-LAMP), is 'n nuwe metode van nukleïensuur opsporing wat voorsiening maak vir vinnige, doeltreffende amplifisering, en visuele bevestiging van aansteeklike agente.

Doel: Die doel van hierdie studie was om 'n nuwe, vinnige en sensitiewe multipleks RSV RT-LAMP toets te ontwikkel wat PoC diagnose van RSV A en B in staat stel.

Metodes: Voorbereiding van 'n kwantitatiewe RSV standaard vir toets optimisering is gedoen met behulp van 'n hipotoniese sel-lise metode van infektiewe virus tydens sub-kultuur, en 'n “shell-vial” kultuur en fluorosensie fokus toets vir titrasie van kultuur-geproduseerde virus voorraad. Ons het 'n enkele stel van agt inleiers ontwerp wat gebaseer is op die groot polimerase geen van beide RSV A en B, en 'n nuwe enkel-stap multipleks RSV RT-LAMP toets ontwikkel, met gebruik van 'n in-huis reaksie mengsel en die Rotor-Gene Q “real-time” thermocycler (Qiagen, Hilden, Duitsland). Die metaalioon aanwyser hidroksi naphtol blou (HNB) is bygevoeg in die multipleks RSV RT-LAMP toets vir visuele bevestiging van RSV.

Resultate: Die finale geoptimiseerde multipleks RSV RT-LAMP toets het 'n analitiese sensitiwiteit van <10 fokus vormende eenhede (FFU) per reaksie vir beide RSV A en B gehad, met 'n gemiddelde tyd tot positiwiteit van 21.85 minute (95% CI 19.2-24.5 minute) , in vergelyking met 90-120 minute vir konvensionele PCR. Geëvalueer teen die Seeplex RV15 multipleks PCR (Seegene, Seoul, Korea) deur 44 (22 RSV A/22 RSV B) nasofaringeale monsters te toets, het die multipleks RSV RT-LAMP toets 'n sensitiwiteit van 100% getoon, en 'n spesifisiteit van 100% wanneer getoets teen nege algemene respiratoriese virusse. Visuele bevestiging van RSV met gebruik van HNB as kolorimetriese reagens was gelykstaande aan die analitiese sensitiwiteit (10 FFU/reaksie) en spesifisiteit (100%) van die multipleks RSV RT-LAMP toets.

Gevolgtrekking: In vergelyking met konvensionele PCR, het ons nuwe enkel-stap multipleks RSV RT-LAMP toets uitstekende sensitiwiteit, spesifisiteit, en wanneer dit gekombineer word met HNB kleurstof kon dit akkurate visuele diagnose voorsien binne 1 uur. Ons verwag dat hierdie multipleks RSV RT-LAMP toets gebruik sal word vir vinnige en sensitiewe RSV bevestiging by die PoC.

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List of Abbreviations:

Abbreviation	Description
ABC	antibiotic cocktail
AGE	agarose gel electrophoresis
ALRTI	acute lower respiratory tract infection
AMPV	Avian Metapneumovirus
ATCC	American Tissue Culture Collection
ATV	activated trypsin-versene
BLAST	Basic Local Alignment Search Tool
CI	confidence interval
CPE	cytopathic effect
CV	coefficient of variation
DFA	direct immunofluorescence assay
DIP	defective interfering particle
DMSO	dimethyl sulfoxide
dsDNA	double-stranded DNA
EIA	enzyme immunosorbent assay
FBS	foetal bovine serum
FFU	focus forming unit
FITC	fluorescein isothiocyanate
F-T	freeze-thaw human
HEp-2	Human epithelial type 2
HIV	human immunodeficiency virus
HNB	Hydroxy naphtol blue
ICU	intensive care unit
IDT	Integrated DNA Technologies
IMF	indirect immunofluorescent
LAMP	loop-mediated isothermal amplification
mAb	monoclonal antibody
MDCK	Maiden Darby canine kidney
MEM	minimum essential medium
NA	nucleic acid
NASBA	nucleic acid sequence-based amplification
NAT	nucleic acid test
NINA	non-instrumented nucleic acid

Abbreviation	Description
PBS	phosphate-buffered saline
PFU	plaque forming unit
q-PCR	quantitative real-time PCR
RE	restriction enzyme
RPMI	Roswell Park Memorial Institute
RSV	Respiratory Syncytial Virus
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SDA	strand displacement amplification
SV	shell vial
TAT	turnaround time
TCF	tissue culture flask
$TCID_{50}$	tissue culture 50% infectious dose
Tm	melting temperature
TMA	transcription-mediated amplification
VTM	viral transport media

Chapter 1: Introduction

1.1 Background

Globally, the burden of disease from respiratory infection exceed that of all other cause of illness.[1] Acute lower respiratory tract infection (ALRTI) contributes substantially to the public health burden of respiratory infections, and unlike mild self-limiting upper respiratory infections such as rhinitis or pharyngitis, often requires hospitalisation. ALRTI affects all age groups, but is more prevalent during early childhood compared to adulthood,[2] and is the leading cause of paediatric morbidity and mortality;[3, 4] it poses a significant challenge to accomplishment of Millennium Development Goal 4 –to reduce child mortality by two thirds by 2015.[5]

An important respiratory viral pathogen is Respiratory syncytial virus (RSV); it is the primary aetiological agent associated with severe ALRTI in infancy and young children worldwide,[6] and accounts for the major proportion of ALRTI-associated hospitalisations within the first year of life.[7]

1.2 Virus properties

RSV is part of the *Paramyxoviridae* family and a member of the genus *Pneumovirus*; two distinct genogroups have been identified, RSV group A and B, based on their respective antigenicity.[8] The RSV genome is non-segmented, with a single strand of negative-sense RNA molecule, approximately 15.2 kb in length, forming an enveloped nucleocapsid and has 10 genes that encode 11 major viral proteins.[9] Three of these polypeptides are trans-membrane envelope glycoproteins (G, F and SH); the heavily glycosylated G protein is responsible for viral attachment to the cell, whilst the F protein mediates cell-to-cell fusion; the G and F proteins have been identified as the primary components to exhibit protective antigenic properties, and are thus of clinical significance.[10]

The structural proteins within the nuclear capsule include: the large RNA polymerase (L), the nucleoprotein (N), and the phosphoprotein (P); collectively these proteins comprise the RSV replicase.[11] A structural, non-glycosylated matrix protein (M), is thought to play an important role in viral budding through the association between the nucleocapsid and viral envelope.[12] Lastly, two non-structural proteins (NS1 and NS2), accumulating only in small amounts during infection, appear to possess an ancillary function of down regulating viral RNA synthesis by inhibition of host type 1 interferon induction.[13]

1.3 Pathogenesis

The incubation period of RSV is approximately 3-5 days, and viral transmission occurs mostly through the nose by inhalation of large aerosolised nuclei, or through hand-to-eye contact with infectious secretions.[14] Early onset of illness is characterised by upper respiratory tract infection, with viral replication in the nasopharynx, followed by migration to the lower airways, showing a particular tropism for the bronchiolar epithelium.[15] Unlike many viruses, infectious RSV virions spread from cell to cell, independent of the extracellular milieu, by inducing cell fusion and forming syncytia – the cytopathic hallmark of infection.[16] This enables the virus to escape the humoral (antibody) immune response, and may explain why RSV is the first respiratory pathogen to affect infants, despite being born with high titres of maternal transplacentally-acquired antibodies.[17]

Infection is restricted to polarised, superficial ciliated cells,[18] entering via the apical membrane,[19] and initially leads to peribronchiolar inflammation with lymphocytes, which progresses to necrosis and desquamation of the bronchiolar epithelium.[20] As infection develops, the resultant necrotic cell debris mixes with increased airway mucosal exudate, forming thick intraluminal plugs, causing the typical pathology of airway blockade and hyperinflation associated with ALRTI.[21]

1.4 Epidemiology

The nidus of RSV infection predominates in humans who are the only natural host, although RSV infection and severe disease in non-human primates has been observed on occasion.[22] Infection by RSV is highly contagious and previous exposure to RSV does not confer immunity to multiple infections recurring throughout life.[23] Infection has seasonal epidemiology, occurring typically during winter in temperate climates and in the rainy season throughout tropical regions, resulting in an estimated 64 million cases of severe respiratory disease and 160 000 deaths annually worldwide.[24] In addition, shifts in the predominance of antigenic subgroups occur in yearly cycles, with even co-circulation of multiple strains within subgroups common,[25] which further enhances immune evasion by the heterologous strain to antecedently induced protection.[26]

The majority of children under 1 year are infected by RSV and almost all are infected before the age of two.[27] A recent study reported RSV as the cause of 34 million cases of ALRTI in children younger than 5 years globally. The bulk of these ALRTI cases occur in resource-limited settings,[28] with 99% of the more than 66 000 annual deaths of children under the age of 5 being in developing countries.[7]

Whilst the effect of RSV infection in children is well known, there is also growing recognition of the importance of RSV-associated ALRTI in the elderly that seem more frequent in developed countries and in contrast to the greater paediatric cases in less affluent countries, with an annual average of 17 358 deaths of adults older than 65 estimated in the USA alone.[29] Taken together, this contributes to a large and periodic burden on health care infrastructure, with hospitalisation costs per case of RSV estimated at an average of US$5 250.[30]

1.5 Risk factors

There are several important risk factors that affect the severity of RSV disease. The pathology of RSV infection means infants and young children are inherently predisposed to ALRTI due to an immature immune system.[31] Furthermore, prematurity goes together with small and vulnerable airways, low titres of maternally derived RSV neutralising antibody transfer,[32] and insufficient cell-mediated immunity, leading to higher ICU admission and longer hospitalisation,[33] along with a significant increase (15.4%) in the rehospitalisation rate of preterm infants who developed chronic pulmonary disease (mostly bronchopulmonary dysplasia).[34]

High risk populations particularly susceptible to severe ALRTI by RSV are those that underwent immunosuppression therapy from solid organ and hematopoietic stem cell transplantation,[35] or suffer from immunodeficiency as shown by a study in South Africa that highlighted increased mortality in children with human immunodeficiency virus (HIV).[36] The other main epidemiological factors are congenital heart disease,[37] and delayed onset of breastfeeding.[38]

The presence of viral co-infection and its influence on disease severity is controversial with conflicting studies,[39] whilst a recent study described the importance of RSV subgroup in disease severity, reporting increased duration of hospital stay and more frequent fatality in patients infected with RSV A.[40] Seldom studied environmental and other host related risk factors that are associated with variable increase of RSV disease severity and rate of hospitilisation, which require further investigation include: maternal smoking and household smoke pollution,[41] low socioeconomic status,[42] crowded living conditions,[43] and living at altitude.[44]

1.6 Treatment and prevention

Therapy of RSV disease is limited to post-hospitalisation supportive care and palliative measures, usually by oxygen therapy and treatment with the only FDA approved RSV antiviral –Ribavirin. Various alternative treatment strategies have been attempted over the years, such as the use of racemic epinephrine, aerosolised recombinant human DNase or corticosteroids, but clinical testing of these showed no efficacious outcome.[45]

To date, the only form of specific RSV prevention is by systemic immunoprophylaxis, currently administered exclusively for high-risk groups with Palivizumab (Synagis®; MedImmune, Gaithersburg, USA), which is a neutralising monoclonal antibody (mAb) specific to the F protein of RSV.[46] Motavizumab is a 2nd generation mAb Palivizumab-derivative that proved more potent and could be administered at lower doses compared to Palivizumab, however the adverse hypersensitivity observed in patients during trials ultimately led to non-approval of the drug.[47]

A number of promising candidate vaccines for active immunisation, using the approach of reverse genetics technology to target gene deletion of a viral genome, are under development and trial,[48, 49] but clinical efficacy has not yet been established. The central challenge for live RSV vaccine development is to attain a safe balance between attenuation and immunogenicity –a highly important factor when considering the partly-competent infant immune system that lacks appropriate B-cell response and antigen presentation.[50]

The high risk of RSV infection in very young infants, before postnatal vaccination courses would have an effect, makes the option of passive immunity conferred to an infant through maternal vaccination during gestation attractive.[51] Although one vaccine candidate has entered clinical trial,[52] the difficulty of this strategy is the requirement of the vaccine to induce the infant immune system to retain high titres of antibodies as maternal antibodies wane in the neonate. Thus, a successful vaccine that would provide protection to infants and high risk individuals from all age groups remains a priority and a challenge to develop.

1.7 Diagnosis

Effective RSV diagnosis allows for correct treatment or management of patients admitted for severe ALRTI, is associated with shorter periods of hospitalisation, and is paramount to decrease disease morbidity and mortality.[53] Furthermore, timely detection of RSV as cause of ALRTI in patients lowers the number of auxiliary tests, ensuring institution of appropriate treatment which is useful to reduce antibiotic use often unrequired in most cases of viral ALRTI,[54] and through isolation of infected patients prevent nosocomial infections.[55]

Rapid and sensitive RSV detection is also crucial to drive preventative programs, evaluate antiviral vaccine efficacy, and provides a valuable tool for epidemiologic monitoring of RSV spread.[56]

1.7.1 Conventional methods

Cell culture isolation of RSV used to be regarded for many years as the gold standard for detection of RSV infection. However, the growth of RSV in culture is slow and arduous to perform, with time to cytopathic effect (CPE) post-inoculation often only observed after 7 days.[57] In addition, a fair amount of technical expertise is required to ensure correct and efficient virus recovery during the chain from sample collection to culture inoculation. An advantage of the culture method is that it enables subsequent genetic and antigenic molecular investigation when the virus is amplified, which is useful for mutation screening and viral transmission analysis.[58, 59]

Antigen detection of RSV is done by direct immunofluorescence assays (DFA) that use fluorescein-labelled antibody detection in epithelial cells,[60] RSV-specific enzyme-linked antibody capture through enzyme immunosorbent assays (EIA),[61] and chromatographic assays.[62] DFA has the benefit of direct observation of infected cells by microscopy, whilst EIA are easily performed and provide rapid confirmation (approximiately 15 minutes), and are thus generaly undertaken in clinical practice for RSV diagnosis. Despite these advantages, a high rate of misdiagnosis (10-30%) and poor specificity has been reported when antigen detection methods are used alone,[63, 64] and EIA lacks adequate sensitivity for RSV confirmation in older children and adults who possess lower viral titres.[65]

1.7.2 Molecular detection

Over the last decade gene amplification by nucleic acid tests (NATs) has become the new gold standard for the detection of numerous viral agents including RSV, as superior sensitivity and specificity has been proved when compared to viral isolation and antigen detection methods,[66, 67] in addition to improved detection efficacy regardless of the age and quantity of viral shedding in the patient tested.[68, 69]

The most frequently used NATs for detection of respiratory viruses are based on the reverse transcription polymerase chain reaction technique (RT-PCR),[70] with several in-house formats developed amplifying various RSV gene targets.[71, 72] Furthermore, a number of commercially available isothermal amplification techniques have been introduced either using strand displacement amplification (SDA), or transcription-mediated amplification (TMA).[73]

Other PCR methods allow simultaneous amplification and detection of a virus genome in real-time by quantitative real-time PCR (q-PCR). These real-time PCR assays typically employ TaqMan fluorescent probes,[74] scorpion probes,[75] or molecular beacons,[76] and result in a substantial reduction of turnaround time (TAT) to a few hours. Notwithstanding, the undeniable weakness of all these nucleic amplification-based assays, when considering the diagnostic end-to-end process holistically, is the necessity of costly equipment and specialised infrastructure which limit their diagnostic utility at the point of care (PoC), especially in resource-poor settings.

1.7.3 LAMP detection

Loop-mediated isothermal amplification (LAMP) is a gene-specific isothermal nucleic acid amplification method developed by Notomi et al. (2000). Unlike PCR, which denatures double-stranded DNA (dsDNA) with heat, strand separation of dsDNA in LAMP is performed by enzymatic activity of a *Bst* DNA polymerase. The high degree of specificity and excellent sensitivity of the LAMP method is attributed to the use of 4 main primers (2 inner and 2 outer primers) designed to recognise six distinct regions of the target sequence, with two primers consisting of complementary sequence in order to make loop structures that initiate self-elongation and subsequent continual strand displacement, generating rapid exponential amplification that yields significant total DNA synthesis, and can be run at a constant temperature without the use of expensive instrumentation.[77, 78] Addition of two more primers called loop primers further accelerates the reaction speed.[79] Lamp typically provides equal or improved detection limits than that of PCR but with much faster reaction time (within 1 hour),[80, 81] and amplified products can be assessed not only by fluorescent RT-PCR, but through measurement of turbidity,[82] or visual observation of a change in reaction colour.[83]

1.8 Research statement

Taken together, the highlighted advantages of LAMP make it particularly applicable and versatile for efficient and cost-effective RSV diagnosis at the PoC in a low-resource environment. With this in mind, the objective of this study was to develop and optimise a sensitive multiplex LAMP assay for rapid detection of both RSV group A and B, which we hypothesise, will improve PoC diagnosis of paediatric and adult RSV suspects.

Chapter 2: Materials and Methods

2.1 Clinical specimens and viral isolates

The study was laboratory based; nasopharyngeal (NP) specimens were used, collected from patients with symptomatic ALRTI from June 2011 to July 2013 at (Tygerberg Hospital, Cape Town, South Africa), as part of routine laboratory testing by the (NHLS, Tygerberg Hospital). All NP specimens were suspended in 5 ml of viral transport media (VTM) containing Eagle's minimum essential medium (MEM; Lonza, Basel, Switzerland), supplemented with 0.2 ml Penicillin-Streptomycin antibiotic cocktail (ABC; Lonza, Basel, Switzerland), and stored at -80°C for future use at the Division of Medical Virology, Stellenbosch University. Stored residual laboratory viral isolates used in the preparation of viral standards, for optimisation and validation of the RSV RT-LAMP assay, were de-identified and assigned new isolate numbers not related to patient information. This study was approved by the Stellenbosch University Health Research Ethics Committee (N11/09/300).

2.2 Preparation of viral standards

2.2.1 Growth of cell lines

In vitro propagation of RSV is done in Human epithelial type 2 (HEp-2) and Maiden Darby canine kidney (MDCK) cell lines. Continuous HEp-2 and MDCK cell lines to be used for downstream cell culture assays were passaged as follows: (All standard cell culture laboratory measures to prevent contamination were adhered to). Cryogenically preserved HEp-2 and MDCK cell stocks, acquired from the American Tissue Culture Collection (ATCC; Manassas, USA), were resuscitated by rapidly thawing the frozen ampoules at 37°C for 2 minutes, and whole ampoule contents transferred to sterile 50 ml Falcon® centrifuge tubes (Corning, New York, USA).

HEp-2 and MDCK cell suspensions were each prepared in 5 ml of modified MEM solution containing 10% Foetal bovine serum (FBS; Sigma-Aldrich, St. Louis, USA) heat-inactivated at 56°C for 30 minutes , 0.2 ml ABC, 5% Dimethyl sulfoxide (DMSO; Corning, New York, USA), 1X Phosphate-buffered Saline without Ca^{++} and Mg^{++} (PBS; containing 8.5 mM sodium phosphate, 1.5 mM potassium phosphate, and 137 mM NaCl, at pH 7.4), and 200 mM L-glutamine solution in 0.85% NaCl (Lonza, Basel, Switzerland). The cell suspensions were centrifuged at 600 x *g* for 5 minutes to remove cryoprotectant, after discarding of the supernatant, the cell pellets were re-suspended in fresh MEM solution and mixed well by vortexing for 1 minute.

The accurate number of total viable cells was counted using an Improved Neubauer haemocytometer, and cell lines were prepared at a standard final concentration of 15 x 10^4 cells/ml;[84] 30 ml of each cell suspension was inoculated into 75 cm^2 CELLSTAR® tissue culture flasks (TCF; Greiner Bio-One GmbH, Kremsmünster, Austria), and incubated at 37°C for 72 hours to prepare cell monolayers. Growth of monolayers was monitored until 70% confluency reached, upon which the medium was discarded, and replaced with 30 ml of MEM containing 2% FBS for the maintenance of cell monolayers. Once cell monolayers were 90%-100% confluent, TCF were incubated at 33°C until used.

To ensure the continuation of the cell lines, sub-culturing of cell lines was done as follows: old medium was discarded from TCF, monolayers were washed with 10 ml of 1X PBS (Ca^{++} and Mg^{++} free), and incubated for 10 minutes at room temperature with 1 ml of pre-warmed 1X Activated Trypsin-Versene solution (ATV; Sigma-Aldrich, St. Louis, USA) added for cell detachment. Following this cell detachment step, any adherent cells not in suspension were dislodged from TCF surfaces using a Porvair cell scraper (Porvair, Wrexham, UK); cells were then harvested, re-inoculated into new TCF, and incubated as described above.

2.2.2 Shell vial culture

The unavailability of ATCC RSV strains at our laboratory during the cell culture phase of the study, which serve as reference standards from the offset, necessitated the cultivation of RSV from residual viral isolates. As highlighted before, traditional RSV culture is a timely process. Centrifugation-enhanced shell vial (SV) culture is a well-established technique in diagnostic virology laboratories. Compared to conventional culture, the SV assay provides a much-reduced TAT (24-48 hours) for the detection of ALRTI-associated pathogens,[85] correlates well with traditional methods, is specific, and has been shown to be more sensitive for RSV detection.[86] A key feature in the SV assay is the utilisation of combinatory cell lines for virus propagation.[87]

We chose the SV assay for the first step of RSV proliferation, to yield a highly infective initial stock that would ensure effective subsequent culture in larger TCF with minimum passage number, providing optimal final RSV culture stocks used to prepare our viral standards. SV assay was done as follows: A mixed cellular suspension of HEp-2 and MDCK cell lines (CoHM) was prepared in Roswell Park Memorial Institute medium (RPMI-1640; Lonza, Basel, Switzerland), modified exactly as with MEM, from our established HEp-2 and MDCK cell lines, with each cell type seeded at 15 x 10^4 cells/ml, and combined in equal amounts. Sterilin™ 7 ml polystyrene SV tubes (Sterilin, Newport, UK), with a clear round coverslip (Lasec, Cape Town, South Africa) inserted, were inoculated with 1 ml amounts of mixed cell suspension, and incubated at 37°C in upright position for 24 hours.

Semi-confluent SV tubes were aspirated of old medium and 200 µl aliquots of viral isolate supernatant, containing either RSV A or RSV B, were delivered to each vial through a Millex® syringe filter (Merck Millipore, Billerica, USA). The vials were centrifuged at 1600 x *g* for 45 minutes and allowed to adsorb at 37°C for 1 hour. After supernatant removal, 1 ml of MEM maintenance medium (2% FBS) was added to each vial, and inoculated vials were incubated at 33°C on a continuous shaker for 48 hours.

2.2.3 RSV visualisation

Positive RSV growth after SV incubation was determined by indirect immunofluorescent (IMF) microscopy as follows: The supernatant of each SV (1.2 ml) was aspirated and aliquots stored in 2 ml graduated Eppendorf Tubes® (Greiner Bio-One GmbH, Kremsmünster, Austria) at 4°C for downstream use; the coverslips were removed with sterile forceps and rinsed by dipping into a bijou containing 1X PBS, then placed for 20 minutes in a bijou filled with -20°C acetone to fix cell monolayers formed on the coverslips. Once fixed, coverslips were mounted, cells up, on glass microscopy slides (Lasec, Cape Town, South Africa) with Entellan® medium (Merck Millipore, Billerica, USA).

The Light Diagnostics™ Respiratory Panel I Viral Screening and Identification kit (Merck Millipore, Billerica, USA) was used for IMF staining as follows: Provided RSV mouse mAb was applied to the entire coverslip and the slides incubated in a moist 5% CO_2 chamber at 37°C for 30 minutes. Unbound antibody was washed from the slides with 1X PBS containing 1X TWEEN® 20 (in the kit), and one drop of fluorescein isothiocyanate (FITC) labelled goat anti-mouse IgG was applied to each coverslip, with slides incubated for a further 30 minutes at 37°C in a CO_2 chamber. Following FITC incubation, provided mounting fluid was applied to each dry coverslip and slides were covered with a cover glass. Prepared slides, including a negative control from the kit, were read with a ProgRes® ultraviolet (UV) microscope (Olympus Soft Imaging Solutions GmbH, Münster, Germany) at X200 magnification. Cells positive for RSV exhibited the characteristic apple-green fluorescence of FITC, whilst uninfected cells stained a dull red.

2.2.4 TCF culture

Sufficient stocks of RSV A and B for preparation of viral standards were cultivated in TCF as follows: The SV culture aliquots of infectious supernatant, from the viral isolates that presented the most growth of each RSV genogroup, stored previously at 4°C, were added to 15 ml Corning® centrifuge tubes (Corning, New York, USA), mixed well by vortexing for 1 minute, and 10 ml of the respective viral supernatants were inoculated into 75 cm^2 TCF with confluent HEp-2 monolayers readied as before. Inoculated TCF were then incubated at 37°C

for 1 hour, to allow cellular adhesion, after which 20 ml of MEM maintenance medium was added and TCF incubated at 33°C for 7 days.

Infected cell monolayers post-TCF incubation were scraped and the cell suspensions were transferred to 50 ml centrifuge tubes, homogenised by vortexing for 1 minute, after which 5 ml aliquots of the cell suspension were made in 15 ml centrifuge tubes and stored at 4°C for IMF detection. Stock to be used for viral extraction was stored at 4°C, whilst remaining stock was preserved at -80°C. Preparation of microscopy slides for IMF detection was done as follows: The aliquots designated for IMF were centrifuged at 600 x *g* for 10 minutes, after pouring off the supernatant, pelleted cells were washed in 5 ml of 1X PBS by vortexing for 1 minute. The centrifugation step was repeated and the cell pellets were re-suspended in 1 ml of 1X PBS. Approximately 10 µl aliquots of the respective cell suspensions were put onto separate 12-well microscopy slides (Lasec, Cape Town, South Africa), allowed to air dry for 20 minutes, and slides were then fixed in -20°C acetone for 10 minutes. The IMF staining procedure and reading of the slides was carried out as before.

Despite good RSV propagation in SV culture, growth of RSV in culture flasks, sufficient for preparation of viral standards, could not be replicated. To overcome this problem, several measures were subsequently implemented to optimise the cell culture process, so as to attain the desired levels of RSV infectivity in culture flasks.

2.2.5 Cell culture optimisation

Our first approach focused on the optimisation of the SV assay, to increase viral titres of the initial SV culture stock for TCF inoculation even further. This involved the addition of a 2nd line of SV culture that would follow 1st line cultivation. With such an increase of passage, an important consideration during viral sub-passage is the presence of defective interfering particles (DIP).[88] DIP lack the full complement of genes necessary for a complete infectious cycle, consequently they compete with functional virions which inhibits infection, decreasing lot to lot titre reliability and assay reproducibility.[89]

To minimise the effect of potential DIP on our SV stock titres, a limiting dilution method was used as follows: 1st line SV culture and IMF confirmation was done as before, after which limiting dilution series (1:1, 1:10, 1:50, 1:100) of harvested infectious 1st line SV supernatant was made; the highest dilution (1:100) was then inoculated onto 2nd line SV tubes and cultured as before.

Furthermore, RSV is an extremely heat labile virus, with elevated loss of infectivity above 37°C within a day.[90] Sucrose has been shown to be an effective stabiliser of RSV under all conditions;[91] therefore, the recovered viral supernatant after 2nd line SV incubation was

supplemented with an equal amount of 25% sucrose solution (25% sucrose in Milli-Q purified H_2O; Sigma-Aldrich) for improved stabilisation, and stored at 4°C for subsequent TCF inoculation.

As noted before, a primary feature of RSV is the cell-associated nature of its replication cycle, which plays an important role in cell culture. Improved virus titres during sub-passage can be achieved by releasing intracellular infectious virions into the culture medium. The conventional technique used for virion recovery from infected cell cultures is by repeated freeze-thaw (F-T) cycles of the infectious medium.[92] F-T cycles are laborious and reduce viral titres; additionally, a large amount of membrane-bound virions may be lost to cellular waste ensuing from the technique.[93] A recent study employed the use of direct water lysis to induce cell-breakage of avian metapneumovirus (AMPV),[94] by creating a hypotonic environment within the cell medium, leading to an increase of water pressure inside cells and their eventual rupture. This method avoids repeated F-T cycles, is rapid, and resulted in increased virion recovery.

Although this alternative method, to our knowledge, has never been used for RSV cell lysis, since AMPV is similar to RSV in being a non-segmented, single-stranded, RNA virus that belongs to the *Paramyxoviridae* family, this suggested it could be applicable for our purpose of RSV recovery. We used this hypotonic lysis method as a pre-inoculation step for optimal TCF cultivation. The optimised cell lysis protocol was carried out as follows: Stored viral supernatant from 2nd line SV culture was added to 15 ml centrifuge tubes and incubated at room temperature with 5 ml of Milli-Q H_2O for 15 minutes. Cells were lysed by repeated pipetting for 30 seconds per minute of incubation (15 times), after which cellular rests were collected by centrifugation at 3000 x *g* for 15 minutes. The viral supernatant, containing a large quantity of cell-free virions, was transferred to new 15 ml centrifuge tubes, and mixed by vortexing for 1 minute with an equal volume (5ml) of 4% MEM (2 ml of 10% MEM with 3 ml Milli-Q H_2O) to restore original stock tonicity. The virus medium was then treated with 25% sucrose solution as before and stored at 4°C for TCF inoculation.

Lastly, the final step of culture optimisation, involved cultivating RSV in TCF seeded with the CoHM cell line mixture, applied from the successful SV culture method, instead of HEp-2 cells only. Briefly, 75 cm^2 TCF were readied for viral inoculation as before, but prepared with a CoHM cell suspension, with each cell type seeded at 15 x 10^4 cells/ml, and added in equal amounts. TCF were inoculated with stored 2nd line SV supernatants, incubated, and IMF confirmation done as before. After confirmation of successful TCF growth (sufficient for preparation of viral standards), harvested cell suspensions were stabilised with 25% sucrose solution. Final RSV stock to be used for titration and RNA extraction was stored as 1 ml aliquots at 4°C, whilst remaining stock was preserved as 15 ml aliquots at -80°C.

2.2.6 Viral quantification

Accurate and effective viral titration forms the core of a reliable standard utilised in assay development and evaluation. Traditional titration methods have relied on ubiquitous plaque reduction assays or CPE based reduction assays;[95] with titre usually expressed as plaque forming units per ml (PFU/ml) or tissue culture 50% infectious dose per ml ($TCID_{50}$/ml). Such reduction assays for RSV titration are time-consuming (taking at least 5-7 days for CPE observation),[96] require costly reagents, and are cumbersome to perform. Several rapid techniques have been reported; based on monitoring changes in absorbance,[97] colorimetric detection,[98] or viral neutralising antigen expression,[99] which render continuous rather than orthodox quantal data, and use automated equipment with advanced computational software.[100, 101] However, the high throughput nature of these methods limits their generalised use.

Domachowske and Bonville (1998) developed a SV fluorescent-focus assay as an efficient, more timely, and easily performed alternative, that correlates well to standard plaque assays.[102] This method was adopted for our RSV titration purposes as follows: Convalescent foci are problematic at low dilutions; therefore, to find the optimal linear range which would allow accurate quantification, serial two-fold dilutions (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200) were made of the RSV A and B culture-derived stock. The dilutions were prepared in RPMI-1640 medium as 1 ml aliquots, of which 200 µl from each dilution was inoculated onto readied shell vials, incubated and mAb/FITC stained as before. Enumeration of fluorescent cells was done manually by IMF microscopy, each fluorescent focus representing 1 infectious unit of RSV, with the titre expressed as focus forming units per ml (FFU/ml). The SV titration assay was done in triplicate for both RSV genogroups.

2.2.7 RNA extraction and preservation

Extraction of RSV RNA to be used as standards for development and optimisation of the RSV RT-LAMP assay, was performed using the NucliSENS® easyMAG® platform (bioMérieux, Boxtel, The Netherlands), according to manufacturer specifications. The NucliSENS easyMAG is an automated nucleic acid (NA) isolation system, utilising magnetic silica extraction technology,[103] and produces optimally recovered NA extracts, free of amplification inhibitors.[104]

Briefly, two-fold serial dilutions (that mirror the titration dilutions and prevents sample oversaturation) of RSV A and B stock were prepared in MEM maintenance medium as 1 ml aliquots, with 750 µl of each dilution used as input volume. Samples are directly lysed on-board for 10 min in Boom lysis buffer containing 5 M guanidinium thiocyanate, after which 50 µl of silica suspension was added manually to all extraction wells and homogenised

thoroughly for RNA adsorption. The silica matrix then underwent a series of washing steps in buffers with high salt concentration to purify the RNA, followed by a heating step at 70°C to release the RNA from the silica. Lastly, 50 µl of purified RNA from each input sample was eluted, and transferred to clear 0.2 ml Axygen® PCR tubes (Corning, New York, USA) that were kept on ice for same day preservation.

RNAstable® (Biomatrica, San Diego, USA) was used for long term storage of RNA standards, allowing anhydrous preservation at ambient temperature, without use of cold storage, thus preventing RNA degradation associated with freezing. Briefly, 10 µl aliquots (dilutions of 10^0, 10^1, 10^2, 10^3, and 10^4) of extracted RSV A and B RNA were each resuspended in 10 µl of nuclease-free water (Qiagen, Hilden, Germany). The 20 µl suspensions were added to RNAstable 96-well plates, mixed gently by pipetting, placed under a laminar flow cabinet to air dry overnight for 12 hours, and once air dried, samples were stored in a dark, non-humid area. Sample recovery only requires re-suspension with nuclease-free water (10 µl for original concentration), and is ready for downstream assays without further purification.

2.3 Primer design

The LAMP technique uses four specially designed primers that discern six distinct regions on the target RNA;[78] these characteristics make it a highly specific and sensitive method for gene amplification, however, the caveat to this efficient amplification are the challenges regarding primer design. The inherently AT-rich RSV genome,[105] along with limited sequence conservation due to the genetic variability among the two antigenic RSV genogroups,[106] prove problematic for multiplex primer design. Further important considerations that add to the intricacy of LAMP primer design are the varying distances required between primer regions, the primer melting temperature (Tm), and stability of primer ends.

A single set of 8 multiplex LAMP primers (see Table 1) to encompass both RSV A and B were designed to target a highly conserved region on the RSV L polymerase gene. Conserved regions in the genogroups were identified by whole genome alignment of a range of reference strains extending the main RSV group A and RSV group B genotypes. The sequences were retrieved from GenBank (see Appendix Table A1), aligned in Geneious software suite (version 6.1.3; Biomatters Ltd., Auckland, New Zealand), after which a combined RSV genogroup consensus sequence was aligned by ClustalW in Geneious and used for primer design.

Primers for the RT-LAMP assay were designed *in silico* using the Primer Explorer web-based software tool (version 4; Eiken Chemical Co., Tokyo, Japan),[107] and then adjusted manually as necessary. Primer sequences were analysed for secondary structure formation with OligoAnalyzer (version 3.1; Integrated DNA Technologies, Iowa, USA),[108] and on the Basic Local Alignment Search Tool (BLAST, NCBI)[109] to screen for specificity against any regions of similarity to other organisms. Primers were synthesised by Integrated DNA Technologies (IDT, Iowa, USA). Outer and loop primers underwent standard desalting, whilst longer inner primers were purified by polyacrylamide gel electrophoresis (PAGE) as these longer primers are more problematic to bind and require extra purification.

Table 1. Primer set used in the multiplex RT-LAMP assay.

Primer	Tm/GC value	Sequence (5'-3')
RSV_F3	54.4°C/33.9%	AAGCAARTATGTTAGAGAAAGATCTTGG
RSV_B3	52.4°C/34.0%	CTCTGYTTTTTGGTTAAAACTTGTC
RSV_LF	50.9°C/25.0%	TATATTTGATGTCCATTGTATACATGAT
RSV_LB	54.0°C/50.0%	AGAGGACCCACTAARCCAT
RSV_A_FIP (F1c+F2)	67.0°C/45.6%	TGCCACTAGCTATAGTGCTTGTTGTTGTTGGTGTTACATCACCCAG
RSV_B_FIP (F1c+F2)	66.1°C/45.6%	TACCACTGGCTATAGTGCTAGTTGTTGTAGGAGTAACATCGCCAAG
RSV_A_BIP (B1c+B2)	63.9°C/37.7%	TGTCAACAGTTTAACACGTGGTTTTTTCTCTTGTGTAGATGAACC
RSV_B_BIP (B1c+B2)	64.4°C/38.2%	TGTTAATGGTTTAACTCGTGGTGAATTTTTCTCCTGCGTAGATGAAC

GC: guanine-cytosine; F3: forward outer primer; B3: backward outer primer; LF: loop forward primer; LB: loop backward primer; FIP: forward inner primer consisting of a forward region (F2) and a complement region (F1c); BIP: backward inner primer consisting of a backward region (F2) and a complement region (B1c).

To accommodate for sequence diversity across RSV genogroups, degenerate bases were used in place of genetically variable bases where necessary. Special attention was given to adjust the Tm of primers in the optimal LAMP order i.e., FIP/BIP > F3/B3 > LF/LB. The approach with regards to sequence mismatches in the inner primers (FIP and BIP), was to design separate inner primers for RSV A and B respectively, so as to mix in a 50:50 ratio; this allows matching of one 'degenerate' with group A and the other with group B, and as the designs are very similar except for a few bases, primer dimer reactions would be limited. Figure 1 illustrates the multiplex RT-LAMP design.

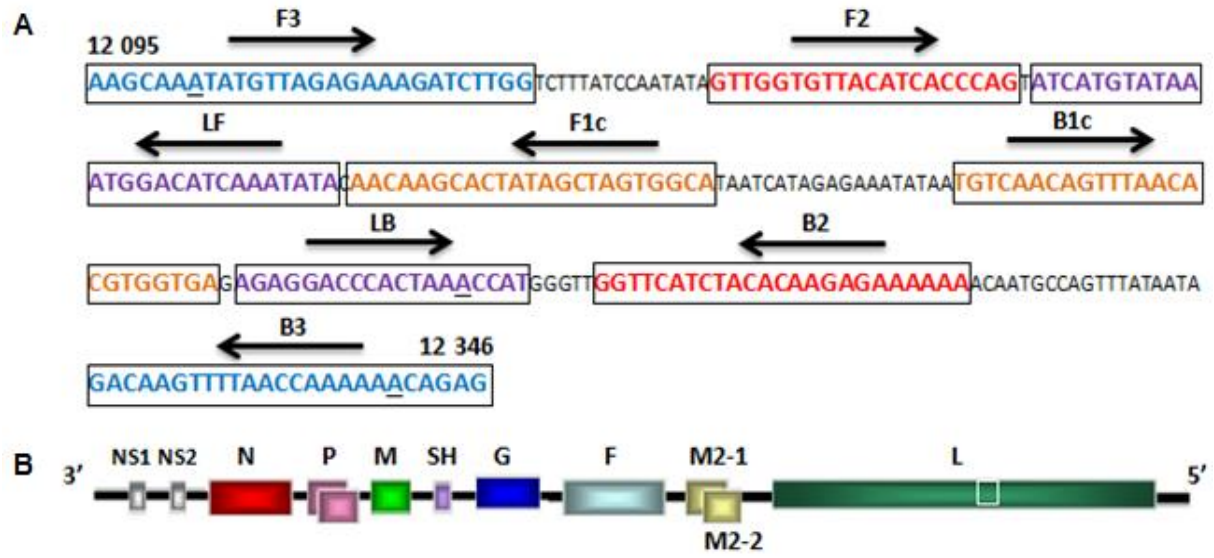


Figure 1. Positioning of primers in RSV genome. (A) Primers are indicated by boxed sequence; primer colour is identical to primers in Table 1; black arrows indicate direction of synthesis; and underlined bases are mismatches in sequence alignment. Template derived FIP is a hybrid primer consisting of a complementary alignment (F1c) which is linked to F2, and initiates strand elongation by converting RNA to cDNA; cDNA amplification occurs likewise with the BIP (B1c and B2). Strand displacement cDNA synthesis is primed by outer (F3) primer, producing dsDNA. BIP and B3 primers then hybridise to the FIP template cDNA, which produces a stem-loop structure. The loop primers (LF and LB) attach to the formed loop and continue DNA synthesis by displacement of the stem-loop. Subsequent LAMP cycling involves the FIP binding to the loop product, displacement DNA synthesis progresses, resulting in the original stem-loop and an additional longer stem-loop. (B) Schematic diagram (not to scale) depicting genes in the RSV genome; block length is in proportion to gene length; and the white box indicates the approximate location of the RT-LAMP primer region in the L gene. Image redrawn using Geneious software suite (version 6.1.3; Biomatters Ltd., Auckland, New Zealand).

2.4 RT-LAMP reaction

To obtain optimal reaction conditions specific for the multiplex RT-LAMP primer set, reagent concentrations and reaction temperatures were tested as optimisation factors. To save costs, an optimised (reagent concentrations adjusted as necessary) in-house reaction mixture (see Table 2) was used for all RT-LAMP reactions, instead of the well-known Loopamp® RNA Amplification Kit supplied by Eiken (Eiken Chemical Co., Tokyo, Japan), that is widely used in LAMP orientated research. Reagent preparation, addition of RNA, and amplification were carried out in separate rooms with restricted access and unidirectional workflow. Throughout optimisation and validation, each RT-LAMP assay included a non-Template Control (NTC) to screen for contamination. All precautions to prevent cross-contamination were observed.

Optimisation of reaction temperature and primer concentration was done by running the RT-LAMP assay at a range of temperatures (59°C, 60°C, 61°C, 62°C, 63°C, and 64°C), and final primer concentrations either at (FIP/BIP: 0.4 µM; LF/LB: 0.2 µM; F3/B3: 0.05 µM) or (FIP/BIP: 0.8 µM; LF/LB: 0.4 µM; F3/B3: 0.1 µM) in the Veriti® 96-well thermal cycler (Applied Biosystems Inc., California, USA). The separate inner primers for RSV A and B were combined in a ratio of 50:50 and added in equal amounts to each reaction. All RT-LAMP assays were subsequently run at 64°C for 60 minutes (30 second cycles), followed by an inactivation step at 80°C for 5 minutes. The RT-LAMP was carried out as a single-step, close-tubed reaction, at a final reaction volume of 25 µl in clear 0.2 ml PCR tubes (Corning, New York, USA).

Table 2. Optimised reaction conditions for RSV multiplex RT-LAMP.

Reagent		Final Concentration	Function
Primers:	**FIP**	**0.8 µM**	
	BIP	**0.8 µM**	
	LF	**0.4 µM**	
	LB	**0.4 µM**	
	F3	**0.1 µM**	
	B3	**0.1 µM**	
2X Reaction mix:			
ThermoPol™ Buffer:		**40 mM Tris-HCl** **20 mM $(NH_4)_2SO_4$** **50 mM KCl** **0.2% Tween-20**	**Isothermal amplification polymerase buffer**
$MgSO_4$		**16 mM**	**Used for optimum polymerase activity**
DNTPs		**2.8 mM each**	**Nucleotides for polymerisation**
Betaine		**1.6 M**	**DNA stabilisation**
Enzymes:			
AMV Reverse Transcriptase		**5U/25 µl**	**First-strand cDNA synthesis**
***Bst* 2.0 WarmStart™ DNA Polymerase**		**8U/25 µl**	**Strand-displacement DNA synthesis**
SYBR® Green I		**0.5X/25 µl**	**dsDNA binding fluorescent dye**
Template RNA		**5 µl**	
Nuclease-free H_2O		**Add to 25 µl**	

ThermoPol Buffer and *Bst* 2.0 WarmStart DNA Polymerase: (New England BioLabs Inc., Ipswich, USA); $MgSO_4$: (Sigma-Aldrich, St. Louis, USA); DNTPs: (Bioline, London, UK); Betaine: (Sigma-Aldrich, St. Louis, USA); AMV Reverse Transcriptase: (Promega Corp., Madison, USA); SYBR Green I: (Life Technologies, Carlsbad, USA).

Further RT-LAMP assay optimisation and validation was done in the Corbett Rotor-Gene Q 6000 real-time thermocycler (Qiagen, Hilden, Germany). Amplification products of the RT-LAMP assay were analysed using Rotor-Gene Q software (version 1.7.87; Qiagen, Hilden, Germany), whereby generated reaction curves (read on FAM channel between 470 nm and 510 nm) reflect the relative fluorescent intensity over reaction time of the RNA amplification products.

During optimisation and validation, agarose gel electrophoresis (AGE) was done to confirm RT-LAMP results. Briefly, 10 µl of amplicons were mixed with 2 µl GeneDirex® Novel Juice dye (Biocom Biotech, Centurion, South Africa) supplied in 6X DNA Loading Buffer, and run at 80 V for 50 minutes with the PowerPac Basic Power Supply (Bio-Rad, California, USA) on a 0.8% agarose gel (SeaKem LE Agarose; Cambrex Bioscience, Maine, USA) in 60 ml of 1X SB buffer (containing 10 mM NaOH, 28 mM boric acid, at pH 8.5). An O'GeneRuler™ 1 kb DNA Marker (Fisher Scientific, Pittsburgh, USA) was used for amplicon size reference. A characteristic ladder pattern can be seen in AGE, since amplicons consist of several inverted repeats, ranging in size, of the target sequence on the same strands. Agarose gels were visualised by UV fluorescence on the Alliance 2.7 optic analysis system (UViTec, Cambridge, UK).

2.5 RT-LAMP validation

The optimised RSV multiplex RT-LAMP assay was evaluated using residual laboratory viral isolates, by comparing it to the current standard assay used for respiratory viral diagnostics in the Division of Medical Virology, Stellenbosch University, which is the Seeplex® RV15 ACE multiplex PCR (Seegene, Seoul, Korea). The minimum sample size was calculated as follows: Assuming the Seeplex RV15 as gold standard and accepting a sensitivity of at least 95%, with an alpha of 0.05 and power of 90%, the required sample size for comparison was 32 specimens.

We evaluated the RT-LAMP assay against 44 pre-selected RSV positive (22 RSV A and 22 RSV B) NP specimens tested on Seeplex RV15. All NP specimens were tested in triplicate with respective RSV A and RSV B positive controls (50 FFU/reaction) included during the RT-LAMP assay. The RNA and DNA of NP specimens and viruses that were used throughout validation tests was extracted on the NucliSENS easyMAG system according to manufacturer specifications as previously described.

Analytical sensitivity of the RT-LAMP assay was determined by testing with culture derived RSV A and B RNA standards (concentrations of 5, 10^1, 10^2, 10^3, and 10^4 FFU/reaction) in triplicate (repeated twice). The RT-LAMP assay specificity of the multiplex RSV primer set was assessed by screening in triplicate RSV standard (10^4 FFU/reaction) against a panel of 9 other common respiratory viruses that were positive by Seeplex RV15 and available in our laboratory, including, Influenza A and B virus, Human parainfluenza virus (hPIV-1, hPIV-2, and hPIV-3), Rhinovirus, Adenovirus, Enterovirus, and Coronavirus 229E. To ascertain the reproducibility of the RT-LAMP assay, 3 replicates each of a positive RSV A and RSV B NP specimen (with unknown concentration) were tested across two different RT-LAMP runs, with

the time to positivity read for each specimen. Both reproducibility runs included RSV A and RSV B positive controls (50 FFU/reaction).

2.6 Confirmation of RSV subgroups

Differentiation of RSV subgroups was done by restriction enzyme (RE) digestion of RSV RT-LAMP products as follows: Restriction enzymes were mapped on the RT-LAMP target region using the online NEBcutter tool (version 2.0; New England BioLabs Inc., Ipswich, USA).[110] Figure 2 indicates the recognition site for each RE. RT-LAMP products were purified using the Wizard® SV PCR Clean-Up System (Promega Corp., Madison, USA) according to manufacturer instructions (see Appendix Protocol A1). RSV A amplicons were digested with FastDigest™ *Nla*III and RSV B with FastDigest™ *Hpy8*I (Thermo Scientific Inc., Waltham, USA) in 30 µl reactions (containing 1 µl RE, 3 µl supplied 10X FastDigest buffer, 1 µl purified DNA, and 25 µl nuclease-free H_2O). RE reactions were incubated in a heating block at 37°C for 2 hours, followed by 5 minute incubation at 80°C to inactivate the RE. Digested RT-LAMP products were then separated by AGE and UV visualised as described before, with the exception of using a 2% agarose gel ran at 80V for 2 hours, and an O'GeneRuler™ 100 bp DNA Marker (Fisher Scientific, Pittsburgh, USA) for product size reference.

RSV A **(252 nb)**

AAGCAAATATGTTAGAGAAAGATCTTGGTCTTTATCCAATATAGTTGGTGTTACATCACCCAGTAT**CATG^**TATACAATG
GACATCAAATATACAACAAGCACTATAGCTAGTGGCATAATCATAGAGAAATATAATGTCAACAGTTTAACACGTGGTGA
GAGAGGACCCACTAAAC**CATG^**GGTTGGTTCATCTACACAAGAGAAAAAAACAATGCCAGTTTATAATAGACAAGTTTT
AACCAAAAAACAGAG

RSV B **(252 nb)**

AAGCAAGTATGTAAGAGAAAGATCTTGGTCATTATCCAATATAGTAGGAGTAACATCGCCAAGTATTAT**GTT^CAC**AAT
GGACATTAAATATACAACTAGCACTATAGCCAGTGGTATAATTATAGAAAAATATAATGTTAATAGTTTAACTCGTGGTGA
AAGAGGACCTACTAAGCCATGGGTAGGTTCATCTACGCAGGAGAAAAAAACAATGCCA**GTG^TAC**AATAGACAAGTTT
TAACCAAAAAGCAAAG

Figure 2. Design of RE digestion sites for confirmation of RSV subtypes. nb: nucleobases; location for the recognition sequence of each RE in the RT-LAMP target region is indicated by underlined sequence; and arrows are the defined cleavage site for each RE. (RSV A) Expected fragments: 42 nb, 47 nb, 84 nb, 94 nb (cut-off fragments); the definitive fragment between cut sites was **180 nb**. (RSV B) Expected fragments: 14 nb, 44 nb, 88 nb, 148 nb (cut-off fragments); the definitive fragment between cut sites was **296 nb**.

Additionally, the RE design included the single use of *Hpy8*I for direct digestion of un-purified RT-LAMP amplicons. Briefly, RT-LAMP products were directly digested, but with *Hpy8*I only, and visualised as before. If RSV B then the expected definitive fragment would be 296 nb (fragment between cut sites), else if RSV A then the expected definitive fragment would be 88 nb (fragment from single cut site). This would allow diagnostic subtyping of RSV A and B.

2.7 Visual detection of RT-LAMP

As a final step of assay development, the multiplex RT-LAMP assay was optimised to allow colorimetric-mediated visualisation of RSV with the naked eye. Hydroxy naphtol blue (HNB) is a metal ion indicator that reacts with the large amounts of magnesium pyrophosphate by-product that forms from a positive LAMP product.[82] A recent study reported on the applicability of HNB as colorimetric reagent for LAMP detection, and showed the HNB LAMP method was associated with reduced contamination risk, compared to other LAMP assays using techniques to visualise a reaction result that require opening of tubes, such as for the addition of intercalating dyes, for instance AGE.[111] Furthermore, the easy and inexpensive nature of the procedure makes it versatile for high-throughput diagnostics.

To monitor the RT-LAMP assay colorimetrically, HNB (Sigma-Aldrich, St. Louis, USA) was added to the pre-LAMP reaction mixture, at a final concentration of 120 µM, replacing SYBR Green I in equal volume (0.5 µl/25 µl reaction). By replacing the intercalating SYBR Green I dye with HNB the Rotor-Gene Q thermocycler cannot be used to read sample positivity as HNB does not fall within the absorbance spectrum of the instrument, making a simple water bath ideal for PoC use. The RT-LAMP assay with HNB was tested for analytical sensitivity and specificity by incubation of culture derived RSV A and B RNA standards (concentrations of 10^1, 10^2, 10^3, and 10^4 FFU/reaction), and against the same panel of 9 other respiratory viruses tested during RT-LAMP validation, respectively. The aforementioned samples were incubated in a water bath at 64°C for 50 minutes, after which the reactions were inactivated at 80°C for 5 minutes in a heating block. The post-LAMP reaction tubes were then assessed for positivity by naked eye.

Chapter 3: Results

3.1 Preparation of viral standards

3.1.1 Cell culture

To prepare high-titered viral standards for downstream RT-LAMP assays, RSV was propagated in cell culture under various conditions, modes of cell culture used, and several techniques incorporated in the process, as illustrated by Figure 3.

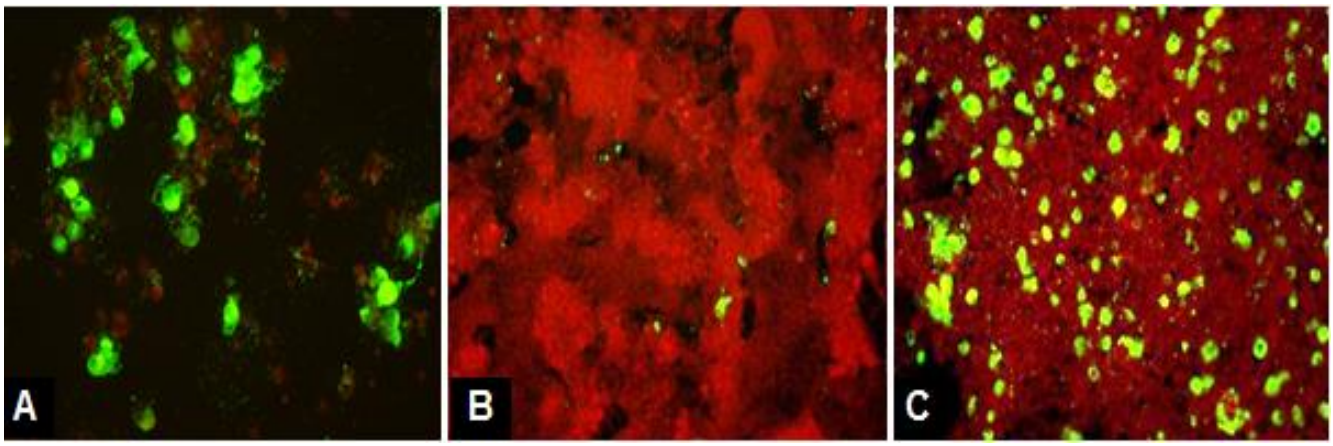


Figure 3. Cultivation of RSV under different culture conditions. Images were captured by UV IMF microscopy; infectious virus was visualised as apple-green fluorescent foci, and uninfected cells stained red; the characteristic syncytia formation of RSV can be seen as large aggregations of positive foci. (A) 2nd line RSV SV culture in CoHM cell line mixture. (B) TCF culture of RSV grown without any optimisation in HEp-2 cell monolayer. (C) TCF culture of sucrose-stabilised RSV, following a pre-inoculation hypotonic cell-lysis step, grown in CoHM cell line suspension.

The centrifugation-enhanced SV technique was used to produce an initial RSV stock, after which adequate quantities of viral stock were cultured in larger TCF. Sufficient RSV proliferation that replicated SV growth could not be achieved in TCF culture, subsequently, a series of steps were implemented to optimise the whole cell culture process.

The optimisation of the SV assay by using a double SV passage approach, and a limiting dilution method to reduce the effect of increased DIP generated during additional passage, yielded high levels of positive RSV growth in SV culture (Figure 3 A). When compared to standard TCF culture pre-optimisation (Figure 3 B), RSV propagation in TCF was significantly improved through the optimised culture procedure (Figure 3 C), ultimately providing the desired high-titre RSV final culture stocks.

3.1.2 Viral quantification

Titration of culture-derived RSV standards was done according to a previously published SV IMF assay.[102] Table 3 shows the representative data for the experiment at two-fold stock RSV dilutions from 1:100 to 1:3200. At the concentrated RSV dilution (1:1) florescent foci were too numerous to count, as we suspect infectious foci might be coalescing, leading to falsely low-titre results.

Table 3. Shell vial fluorescent-focus titration assay of RSV standards.

SV No.	Direct FFU per SV	Dilution Factor	FFU/ml	Avg. FFU/ml/Dilution*	
A1	TMTC		TMTC	1:1	TMTC
A2	1 022	100	511 000		
A3	404	200	404 000	1:100	463 500
A4	287	400	574 000		
A5	173	800	692 000	1:200	448 667
A6	91	1 600	728 000		
A7	38	3 200	608 000	1:400	520 667
AA1	TMTC		TMTC		
AA2	956	100	478 000	1:800	589 333
AA3	338	200	338 000		
AA4	269	400	538 000	1:1600	680 000
AA5	181	800	724 000		
AA6	101	1 600	808 000	1:3200	688 000
AA7	42	3 200	672 000		
AAA1	TMTC		TMTC		
AAA2	803	100	401 500		
AAA3	604	200	604 000		
AAA4	225	400	450 000		
AAA5	88	800	352 000		
AAA6	63	1 600	504 000		
AAA7	49	3 200	784 000		
B5	296	800	1 184 000	1:800	1 240 000
B6	145	1 600	1 160 000		
B7	72	3 200	1 152 000	1:1600	1 120 000
BB5	311	800	1 244 000		
BB6	132	1 600	1 056 000	1:3200	1 146 667
BB7	69	3 200	1 104 000		
BBB5	323	800	1 292 000		
BBB6	143	1 600	1 144 000		
BBB7	74	3 200	1 184 000		

TMTC: too many to count; SV number represents respective RSV A and B replicates at each dilution which was done in triplicate; * average fluorescent focus units/dilution x dilution factor x 5 fluorescent focus units/ml (200 µl per SV).

The optimal linear range which would allow accurate quantification of RSV culture standards was found to be at the two highest dilutions (indicated by red blocks in Figure 4), since there was no confluence of fluorescent foci or other factors resulting in lower titres. This might be due to the competition factor being minimised at high dilutions, and as these yielded higher titres with the lowest difference in absolute value (8000 FFU/ml for RSV A and 26667 FFU/ml for RSV B), reading at these concentrations is likely accurate. The high r^2 value (0.9273) indicates the goodness-of-fit of the linear regression data for the experiment.

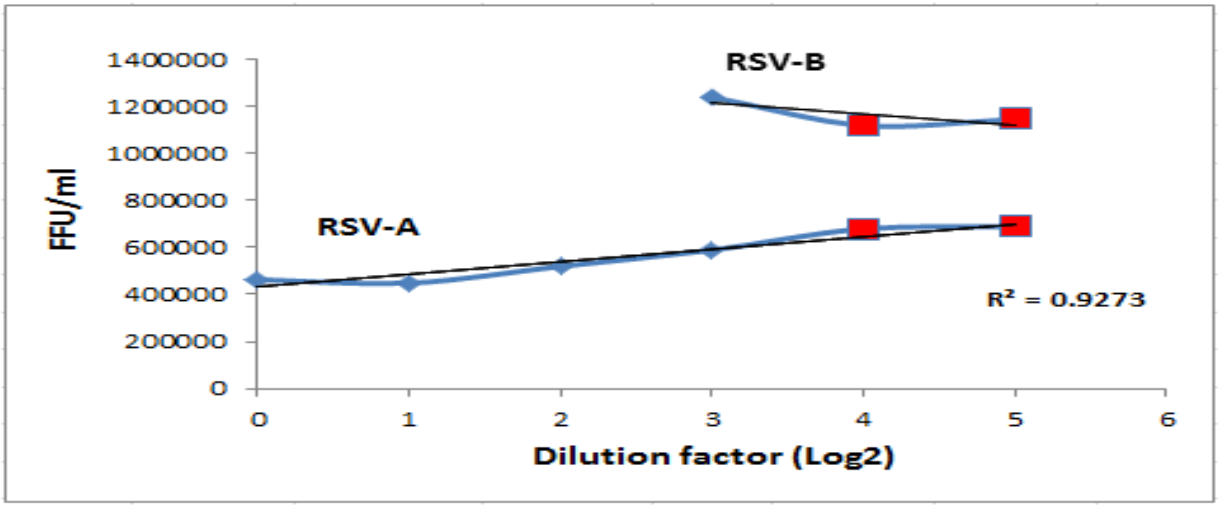


Figure 4. Lines of unity and scatter plots of RSV SV titration assay. Data of fluorescent focus units/ml is of the available 18 RSV A and 9 RSV B dilution replicates; red blocks are the data points reflecting the two highest dilutions of respective linear sets.

As the optimal linear range of quantification was shown to be at the highest dilutions in the RSV A titration assay, only the three highest dilutions (1:800, 1:1600, and 1:3200) were used for RSV B titration.

3.2 RT-LAMP reaction

The RT-LAMP assay was optimised by testing the designed multiplex primer set with an in-house reaction mix under different reaction conditions, with results confirmed by AGE (see Figure 5).

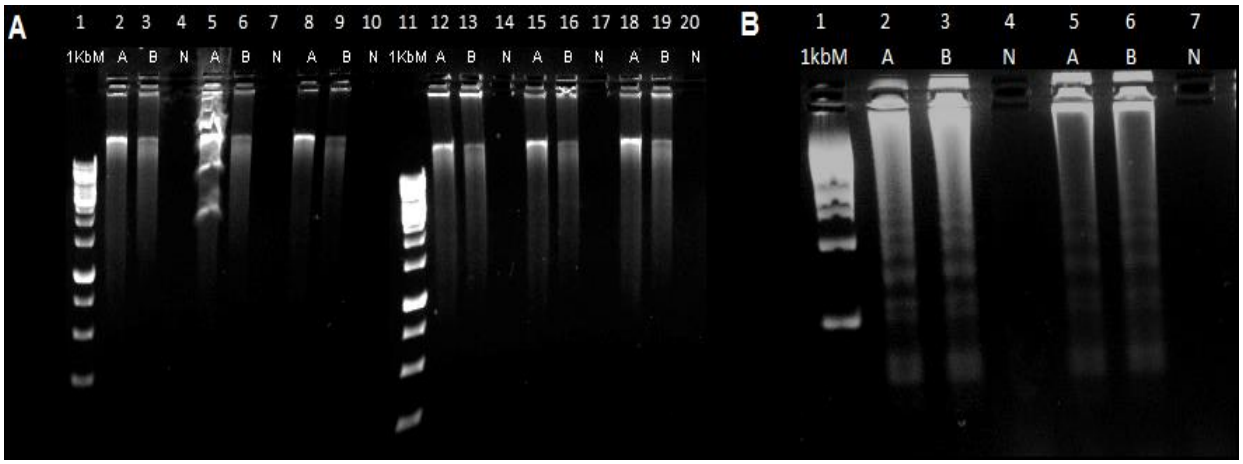


Figure 5. Testing of optimisation factors in the RT-LAMP assay. A: RSV A standard (10^3 FFU/reaction); B: RSV B standard (10^3 FFU/reaction); N: non-Template control. (A) Reaction temperature optimisation; Lane 1: 1 kb O'GeneRuler DNA marker; Lane's 2-4: 59°C; Lane's 5-7: 60°C; Lane's 8-10: 61°C; Lane's 12-14: 62°C; Lane's 15-17: 63°C; Lane's 18-20: 64°C. (B) Primer concentration optimisation at 64°C; Lane 1: 1 kb O'GeneRuler DNA marker; Lane's 2-4: (FIP/BIP: 0.8 µM; LF/LB: 0.4 µM; F3/B3: 0.1 µM); Lane's 5-7: (FIP/BIP: 0.4 µM; LF/LB: 0.2 µM; F3/B3: 0.05 µM).

The RT-LAMP multiplex primer set successfully amplified template RSV RNA with the in-house reaction mix across all annealing temperatures (Figure 5 A) and primer concentrations (Figure 5 B). The optimal annealing temperature was deemed to be 64°C, and a final primer concentration at (FIP/BIP: 0.8 µM; LF/LB: 0.4 µM; F3/B3: 0.1 µM), as these reaction conditions produced the highest amount of amplified product (see Figure 5), and shortest reaction time (see Figure 6).

Figure 6 illustrates visualisation of time to positivity for amplification products on the Corbett Rotor-Gene Q, and represents the experiment to optimise RT-LAMP primer set concentration. The instrument was programmed to use cycles of 30 seconds as reaction time as this provides a more efficient acquisition of fluorescence data for the LAMP reaction. Positive samples could be clearly distinguished from negative samples, as reaction curves of positives exhibit a distinct exponential phase of amplification, compared to the continual lag phase of negative reaction curves with non-specific fluorescence well below the plateau of positives (see Figure 6).

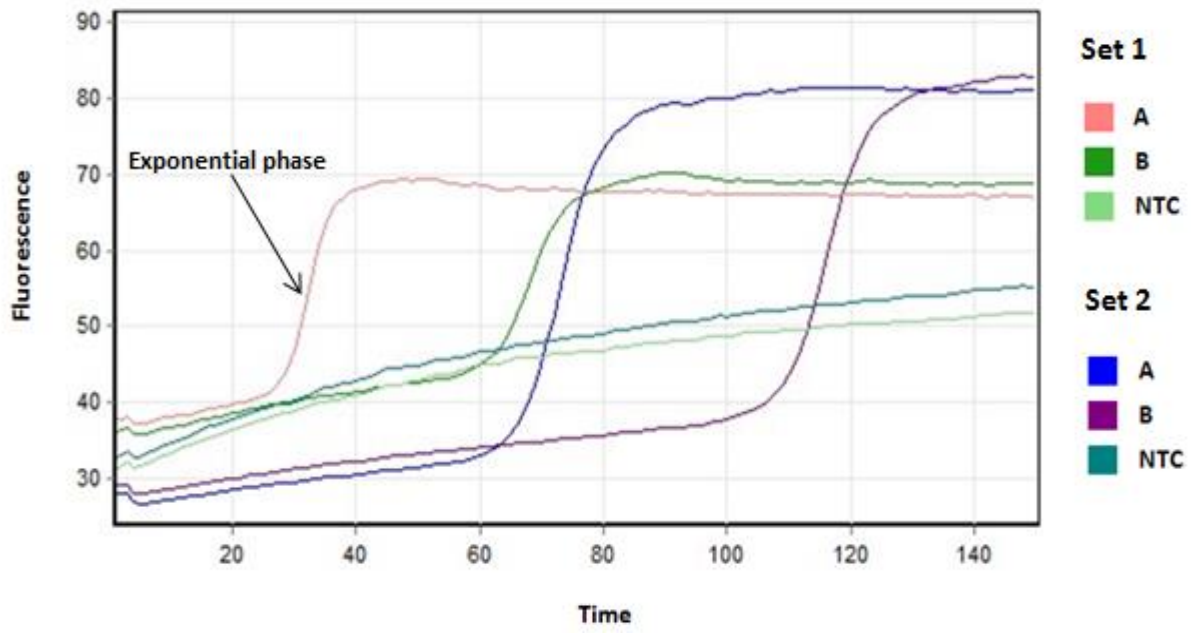


Figure 6. Real-time reaction curves of RT-LAMP primer concentration optimisation. The *x*-axis refers to reaction time (**cycles of 30 seconds**), and the *y*-axis refers to relative fluorescent intensity; colours of the reaction curves correspond to sample legend; RT-LAMP reaction was run at 64°C; A: RSV A template (10^3 FFU/reaction); B: RSV B template (10^3 FFU/reaction); NTC: non-Template control; Set 1: Primer concentration at (FIP/BIP: 0.8 µM; LF/LB: 0.4 µM; F3/B3: 0.1 µM); Set 2: Primer concentration at (FIP/BIP: 0.4 µM; LF/LB: 0.2 µM; F3/B3: 0.05 µM). Time to positivity: Set 1-A (13 minutes) vs. Set 2-A (32 minutes), Set 1-B (30 minutes) vs. Set 2-B (52 minutes).

3.3 RT-LAMP validation

3.3.1 Clinical specimen evaluation

The performance of the optimised multiplex RT-LAMP assay to detect RSV was assessed against positive laboratory viral isolates (22 RSV A and 22 RSV B) previously tested by Seeplex RV15 PCR. The data generated by RT-LAMP evaluation is shown in Table 4.

Table 4. Amplification times for RSV positive specimens evaluated by RT-LAMP.

Specimen	Mean Amplification Time (min:sec)	Specimen	Mean Amplification Time (min:sec)
A1	17:00	B1	29:30
A2	11:00	B2	23:00
A3	14:30	B3	29:00
A4	33:45	B4	25:30
A5	30:30	B5	26:00
A6	21:45	B6	31:15
A7	12:30	B7	39:30
A8	15:00	B8	36:30
A9	16:00	B9	48:20
A10	17:00	B10	24:30
A11	13:30	B11	28:45
A12	14:15	B12	30:00
A13	15:00	B13	32:45
A14	13:00	B14	22:00
A15	16:15	B15	21:15
A16	18:30	B16	18:00
A17	20:00	B17	17:30
A18	15:30	B18	17:15
A19	14:00	B19	15:30
A20	13:15	B20	18:00
A21	12:45	B21	25:45
A22	13:00	B22	36:15

Specimens are named according to respective RSV A and B specimens and the number of each tested (22 RSV A and 22 RSV B); mean amplification time is of the three replicates of each positive RSV specimen tested in triplicate by RT-LAMP.

Of the 22 RSV A isolates that were positive by Seeplex RV15, all 22 were positive by RT-LAMP, and of the 22 RSV B isolates that were positive by Seeplex RV15, all 22 were positive by RT-LAMP. Thus, the multiplex RT-LAMP assay had a sensitivity of 100% for detecting RSV (44/44). The mean amplification time for the 22 pre-selected RSV A positives was 16.7 minutes (ranging between 11 and 33.75 minutes; 95% CI 14.2-19.1 minutes), and the mean amplification time for the 22 pre-selected RSV B positives was 27 minutes (ranging between

3.3.3 Primer specificity

The RSV RT-LAMP primer set specificity was screened in triplicate against residual laboratory isolates of 9 other common respiratory viruses that were positive by Seeplex RV15 PCR. According to the results of the RT-LAMP, the multiplex RSV primer set only amplified RSV template, and did not react with other respiratory viruses (see Figure 9). Therefore, the multiplex RT-LAMP assay was 100% specific for detecting RSV.

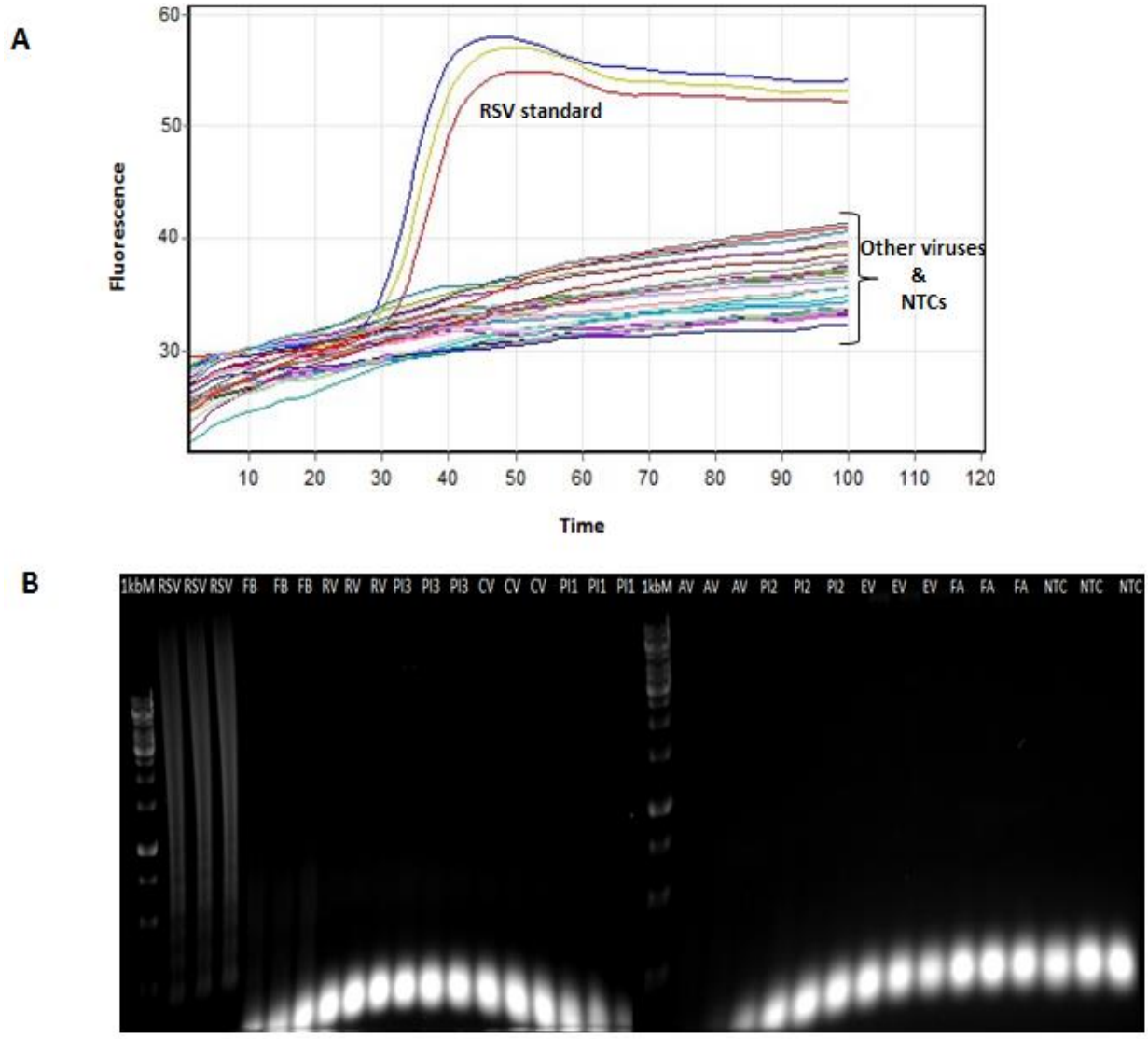


Figure 9. Reaction specificity of multiplex RT-LAMP primer set. Other viruses are the non-RSV respiratory viruses which include FB: Influenza B; RV: Rhinovirus; PI3: Parainfluenza-3; CV: Coronavirus 229E; PI1: Parainfluenza-1; AV: Adenovirus; PI2: Parainfluenza-2; EV: Enterovirus; FA: Influenza A. NTC: non-Template control. (A) Real-time differentiation of RT-LAMP primer specificity; the *x*-axis refers to reaction time (**cycles of 30 seconds**), and the *y*-axis refers to relative fluorescent intensity; RSV standard is 10^4 FFU/reaction. (B) Confirmation of real-time results by AGE; 1kbM: 1 kb O'GeneRuler DNA marker; RSV: RSV standard (10^4 FFU/reaction).

3.3.4 Reproducibility

The repeatability of the RT-LAMP assay was determined by testing 3 replicates over two separate RT-LAMP runs for each RSV subgroup from respective NP specimens that were positive by Seeplex RV15 and subsequently RT-LAMP. Time to positivity data of the experiment is shown in Table 5.

Table 5. Reproducibility of RT-LAMP testing.

Specimen	Amplification Time (min:sec)	
	Run 1	Run 2
A1	14:00	14:30
A2	13:15	14:00
A3	15:15	15:00
B1	35:45	42:30
B2	34:15	39:45
B3	37:30	43:00

Specimens are named according to respective RSV A and B replicates and the number of each tested.

The mean amplification time for the 3 RSV A replicates across both runs was 14.33 minutes (with amplification times within 2 minutes for each replicate; mean SD 0.74 minutes), and the mean amplification time for the 3 RSV B replicates across both runs was 38.79 minutes (with amplification times within 8.75 minutes for each replicate; mean SD 3.58 minutes).
The coefficient of variation (CV) for the respective subgroup replicates was very low and similar (5.1% for RSV A and 9.2% for RSV B), suggesting the multiplex RT-LAMP assay was highly reproducible for both RSV subgroups.

3.4 Confirmation and subtyping of RSV subgroups

The RSV subgroups were classified by RE digestion of RT-LAMP products. RSV A amplicons were digested with FastDigest *Nla*III and RSV B with FastDigest *Hpy8*I. Furthermore, the RE design was such that *Hpy*8I could be utilised exclusively for direct digestion of un-purified RT-LAMP amplicons, which would enable diagnostic subtyping of RSV A and B. Results of the RE digestion experiment were confirmed by AGE (see Figure 10).

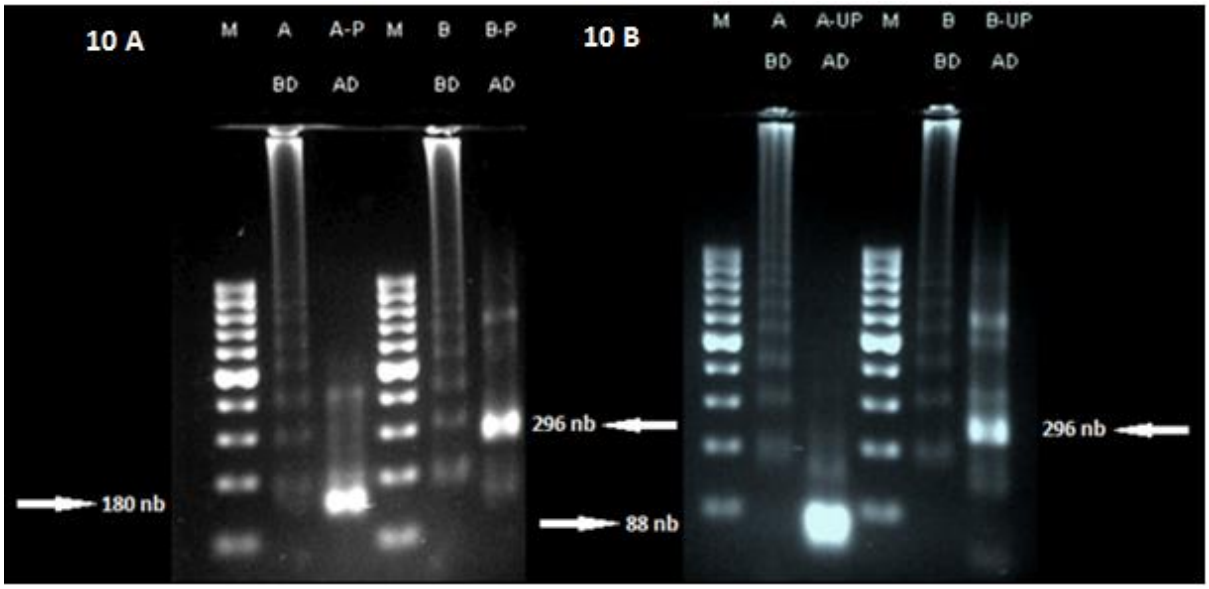


Figure 10. Electrophoresis of RT-LAMP products after RE digestion. M: O'GeneRuler 100 bp DNA marker; A: RSV-A; B: RSV-B; A-P: RSV-A Purified; B-P: RSV-B Purified; A-UP: RSV-A Un-purified; B-UP: RSV-B Un-purified; BD: Before Digestion; AD: After Digestion; nb: nucleobases; white arrows indicate size reference of definitive bands for respective digestions.

Un-digested RT-LAMP products presented with the prominent ladder pattern of DNA amplicons, whilst they formed one major band with smaller minor bands post-digestion. The expected 180 nb definitive fragment, specific for *Nla*III RSV A digestion, was clearly distinguishable from the expected 296 nb definitive fragment specific for *Hpy8*I RSV B digestion (see Figure 10 A). RSV subgroup differentiation was possible from un-purified digestion using *Hpy8*I only, with RSV A forming a definitive band of 88 nb, compared to the 296 nb definitive band of RSV B (see Figure 10 B).

3.5 Visual detection of RT-LAMP

Hydroxy naphtol blue was added to the pre-LAMP reaction mixture as colorimetric indicator to allow for visual diagnosis of RSV by RT-LAMP in a water bath. The run time of the HNB RT-LAMP assay should be set at 50 minutes, since it corresponds to the upper range of time to positivity determined in clinical specimen testing, followed by 5 minutes inactivation of reaction enzyme to prevent further amplification. The post-LAMP reaction tubes could be monitored for positivity by the naked eye in good ambient light conditions (optimally on a light box), with a positive reaction indicated by a change of violet (pre-amplification) to the characteristic sky blue colour, as illustrated in Figure 11 and Figure 12.

The RT-LAMP assay could detect over four logs of culture derived standard FFU for both RSV subgroups (see Figure 11), and was negative for all 9 other respiratory viruses screened against, whilst only being reactive for respective RSV subgroups (see Figure 12). Thus, visual detection of RSV using HNB as colorimetric reagent was equivalent to the analytical sensitivity (10 FFU/reaction) and specificity (100%) of the multiplex RT-LAMP assay.

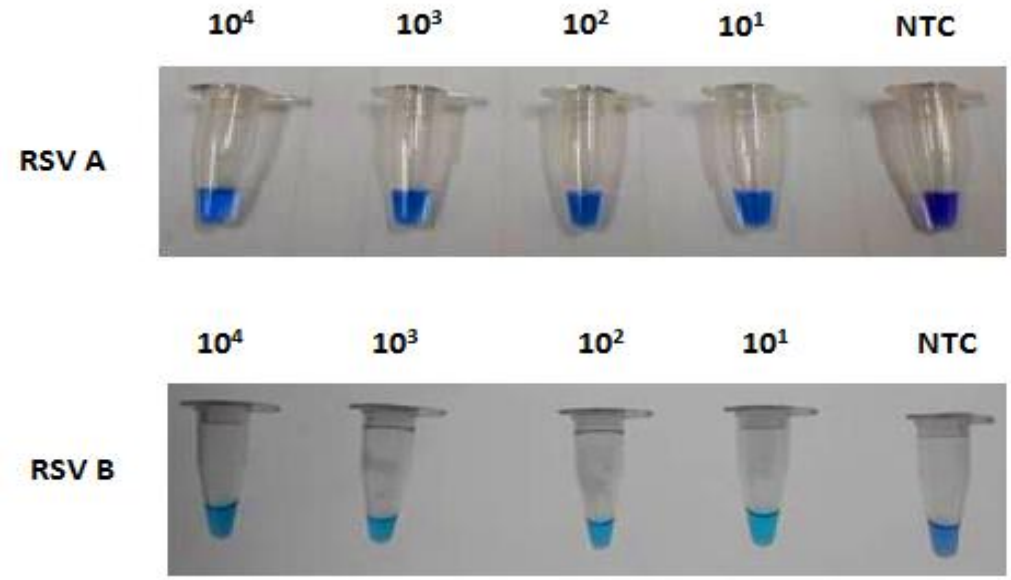


Figure 11. Visual detection sensitivity of RT-LAMP assay using HNB for detection of RSV subgroup A and B standards. NTC: non-Template control; indicated numbers are the RSV standards concentration of 10^n FFU/reaction; the colour changes from violet (negative reaction) to sky blue (positive reaction).

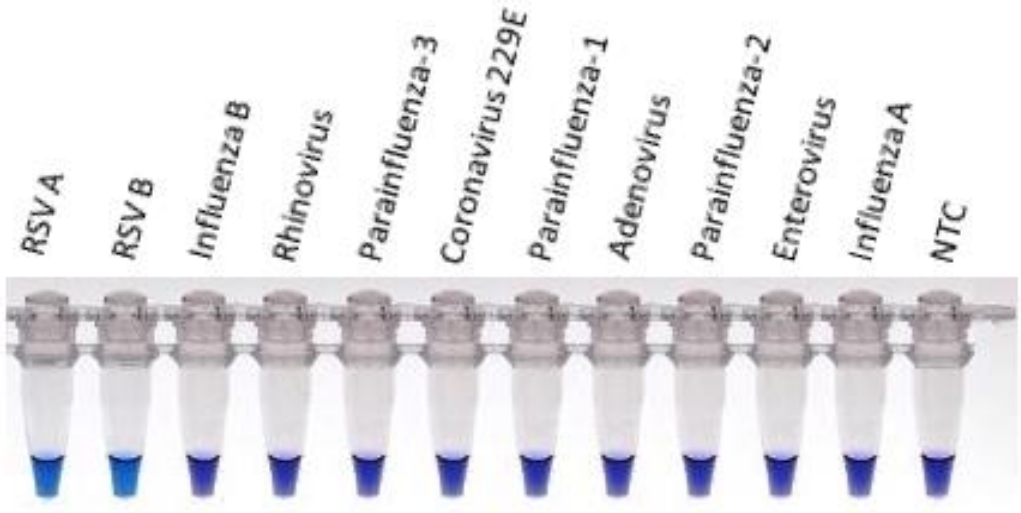


Figure 12. Reaction specificity of RT-LAMP assay with HNB for visual detection. Concentration of RSV A and B standards is 10^4 FFU/reaction; negative reactions are violet colour and sky blue colour indicates positive reactions; NTC: non-Template control.

Chapter 4: Discussion

4.1 Rationale and main finding

The past 10 years has seen conventional methods for RSV detection being replaced by more sensitive molecular methods. Virus isolation is labour intensive and slow to provide results, whilst enzyme or immunofluorescent antigen detection-based tests lack appropriate diagnostic sensitivity and specificity. Routinely used nucleic acid-based molecular techniques such as RT-PCR and q-PCR produce more definitive diagnostic results with faster performance, but require specialised facilities and expensive equipment. RT-LAMP offers a robust and simpler to perform diagnostic alternative, with excellent time to diagnosis, and without the need for costly thermocyclers or fluorescent probes, making it highly versatile and ideal for use at PoC.

This study describes a highly sensitive, specific, and reproducible molecular isothermal diagnostic method for the rapid and simultaneous detection of both RSV subgroups using an optimised, novel RT-LAMP assay with a single multiplex primer set, that can be run in a standard water bath or heating block. The RT-LAMP assay developed in this study had an analytical sensitivity of <10 FFU/reaction, specificity of 100%, and could qualitatively detect RSV in all clinical NP specimens tested (n = 44) within 60 minutes (compared to 90-120 minutes by PCR), with a mean time to positivity for all specimens (RSV A and B) of 21.85 minutes (95% CI 19.2-24.5 minutes).

The RT-LAMP assay was further developed to provide definitive, cost-efficient differentiation and diagnostic typing of RSV subgroups within 4 hours, following direct restriction enzyme (RE) digestion and agarose gel electrophoresis (AGE) visualisation, thereby removing reliance on high-priced methods such as genome sequencing. As final element of assay optimisation, HNB was used to enable easy visual colorimetric-mediated multiplex RSV detection, which matched RT-LAMP analytical sensitivity and specificity, with specific aim towards improving PoC applicability for wide use in resource-limited settings, especially developing countries.

The rapid and accurate detection of RSV provided by the multiplex RT-LAMP assay developed in this study could significantly improve the effective management and treatment of patients, helping to decrease disease morbidity and mortality associated with RSV ALRTI. Furthermore, timely diagnosis would aid public health authorities in drawing up appropriate plans for prevention and infection control strategies to protect high risk individuals during an outbreak, and limit unnecessary or potentially harmful antibiotic use.

4.2 RT-LAMP in context

Despite the advent of isothermal amplification methods being employed more regularly than classical laboratory methods for diagnosis of respiratory viruses since the early 2000's,[112, 113] a limited number of isothermal amplification tests have been developed for RSV detection, with varying clinical and PoC usefulness. A commercial nucleic acid sequence-based amplification (NASBA) assay by bioMérieux, the NucliSENS EasyQ RSV A+B NASBA, has been reported to compare favourably to culture, IF and PCR methods with detection of RSV A and B ranging from 95-20 copies/reaction.[114, 115]

Furthermore, only four reports have described the use of RT-LAMP to detect RSV. Ushio et al. (2005) used separate singleplex RT-LAMP assays to detect the RSV genome targeting the N protein, with amplification taking 1 hour and an analytical sensitivity of 4 and 12 $TCID_{50}$/ml for RSV A and B respectively.[116] However, this study only validated specificity for RSV detection against 3 influenza virus strains. In comparison, we tested our multiplex RT-LAMP primer specificity against a varied panel of 9 other respiratory viruses, with detection of RSV only. In 2007, Shirato et al. could detect 10^2 copies/reaction of RSV A and B from the M gene by singleplex RT-LAMP assays.[117] Both these studies used a limited number of RSV genome sequences, 2 and 6 respectively for primer design, possibly compromising the coverage of RSV detection. In contrast, our RT-LAMP primer design strategy focused on using a range of the most frequently isolated RSV genotypes, with at least 14 different reference strains in order to ensure that the design would enable amplification of diverse RSV strains.

Recently, Mahony et al. (2013) and Mu et al. (2014) developed multiplex RT-LAMP assays for RSV detection.[118, 119] These two reports, together with the abovementioned singleplex-based studies, all used two separate RSV group A and B-specific primer sets of 6 primers each (12 primers in total), with the two multiplex-based studies simply combining primer sets for simultaneous RSV detection. Herein, the multiplex RT-LAMP assay developed in our study differs, since primer design is based on a novel single set of 8 primers targeting the L gene of RSV with only inner primers being a mixture of 2 primer sets, to accommodate divergence between RSV A and B.

This approach of primer design allowed true multiplex detection of both RSV subgroups regardless of the inherent challenges of RSV LAMP primer design (e.g. limited sequence conservation, amount of primers involved, and thermodynamic requirements). The use of a single primer set for concurrent detection of RSV A and B is an important factor for PoC applicability in low-resource settings, as it reduces the cost and simplifies ease of use for the assay.

The analytical sensitivity (<10 FFU/reaction) and rapid amplification (mean amplification time of 21.85 minutes for RSV A and B) of our multiplex RT-LAMP assay were either better than these earlier RSV RT-LAMP assays or comparable (Mahony et al., 2013). A cost-effective in-house reaction mix was used, with the choice of enzyme mix particularly influencing dynamics of RT-LAMP amplification. Heterodimeric AMV-RT is ideal for cDNA synthesis in RT-LAMP, which requires high reaction temperatures, due to its high RNase H activity and intrinsic thermal stability.[120] For optimal DNA strand-displacement, we used the improved *Bst* 2.0 WarmStart DNA polymerase, which amplifies significantly faster with increased yield compared to standard *Bst* DNA polymerase, and its warm start feature solves the problem of undesired enzyme activity during reaction setup.[121]

When evaluating the reliability and utility of a multiplex diagnostic assay, it is important to achieve highly similar analytical sensitivities for respective subgroups. The multiplex RT-LAMP assay in this study had equal analytical sensitivity for both RSV subgroups, whereas Mu et al. (2014) reported a large difference in detection sensitivity (1.58 $TCID_{50}$/ml for RSV B and 281.17 $TCID_{50}$/ml for RSV A). This might result in an amplification bias, which may explain the striking difference in the proportion of NP specimens that tested positive for RSV in their study (of 36 positives sequenced, 11 were RSV A vs. 25 RSV B), which could imply false-negatives in the overall 77 NP specimens tested by their RT-LAMP assay.

Of the 4 previous RSV RT-LAMP studies, only one (Mu et al., 2014) developed visual colorimetric detection for confirmation of positive RSV (using calcein), whilst Ushio et al. (2005) and Shirato et al. (2007) used an expensive real-time turbidimeter and Mahony et al. (2013) measured standard real-time fluorescence in a PCR system. Goto et al. (2009) showed visual detection of LAMP products using HNB to be a simple, easy to operate and diagnostically versatile alternative to other methods, with HNB LAMP assay sensitivity equal (160 λ DNA copies/reaction) to an assay using SYBR Green and superior to a calcein-based assay (10 times lower).[111] Naked eye detection of RSV in our RT-LAMP assay using HNB matched the performance indicators of the RT-LAMP by Rotor-Gene Q fluorescent detection, and results of the HNB assay can be read for at least 2 weeks, as the colours of HNB in positive/negative LAMP reactions remain stable over this time,[111] adding to the suitability for PoC diagnosis.

4.3 RSV standards and secondary findings

A stable and high-titred RSV standard is essential for development, quality control, and ensuring a reproducible diagnostic assay. The thermolability and cell-bound replication of RSV makes *in vitro* culture notoriously slow and difficult. Lack of laboratory ATCC RSV standards required propagation of RSV from older residual viral isolates that had been

subjected to several F-T cycles through previous diagnostic testing, greatly reducing the quantity of infectious particles. A centrifugation-enhanced SV technique was used to produce highly-infectious seed stocks for TCF proliferation and combined with immunofluorescent microscopy for positive RSV visualisation, negating the need for CPE observation and resulting in rapid, cost-effective detection of infectious RSV within 48 hours.

Sufficient RSV growth comparable to SV culture could not be replicated in TCF, necessitating several steps to optimise the cell culture process. By implementing a double-passage SV approach, and using limiting dilutions during sub-passage to nullify the effect of possible DIP, a more potent yield of RSV seed stock for TCF culture was achieved. TCF were inoculated with 25% sucrose-stabilised viral supernatant that was subjected to hypotonic lyses. Hypotonic water lysis of viral supernatant provided simple, effective virion recovery from cells in 15 minutes and averts possible loss of virus titre, as is the case with F-T virus collection which is time-consuming and undergoes regular temperature change.

Furthermore, as an adaptation from the SV assay, RSV was cultivated in TCF in a CoHM cell line mixture, which is thought to be useful for rendering a more accommodating growth environment with respect to different RSV genotypes. The incorporation of these optimisation steps into the culture process significantly improved RSV isolation frequency *in vitro* and produced a stable and high-titred RSV preparation.

Although the SV fluorescent-focus titration method required manual counting of fluorescent foci, this is offset by allowing timely (within 48 hours) and accurate quantification (i.e., high correlation, $r^2 = 0.9273$, of optimal linear regression data) of RSV culture standards, the low cost of reagents involved, making it more useful in resource-poor settings, as opposed to conventional enumeration by laborious reduction assays where culture takes long and CPE is often difficult to observe. Lastly, an important factor is long-term storage of the RNA standards, which was done through anhydrous preservation at room temperature, ensuring RNA integrity was maintained during assay development and validation.

We strongly believe our findings suggest the culture techniques, optimisation process, and titration method used in the cell culture phase of this study, as an integrated approach, could be valuable for preparing reliable, quantified viral standards that can be used for the optimisation of molecular assays. In addition, this approach could lend itself to modification for culture of other paramyxoviruses and cell-associated viruses that are known to be difficult to isolate in the laboratory.

4.4 Study limitations

The high rate of nucleic acid amplification products generated by the LAMP reaction, which results in the excellent sensitivity and specificity associated with the method, does come with some inherent caveats to consider. The LAMP technique requires careful contamination control and hence a certain level of training; this may be addressed through standard good laboratory practice, such as inclusion of NTCs during assay runs and unidirectional workflow throughout reaction setup. Furthermore, the use of AMV RT and addition of HNB pre-amplification permits single-tube RT-LAMP, thus allaying concerns of cross-contamination. The high amplification nature of LAMP may also be associated with background amplification, which necessitates the assay to be stopped at a determined time to ensure prevention of false positives. This may be a superficial limitation however, easily negated by a well-established reaction cut-off time based on assay validation (as in this study), and its function as a rapid diagnostic assay.

We did not validate the multiplex RT-LAMP assay against rare RSV genotypes, but as our primers virtually cover the full spectrum of RSV through an approach of targeting several reference strains, added to the use of degenerate bases that allows flexibility to molecular variation, it is likely that our multiplex primer set will bind these outlier genotypes. Lastly, since old residual laboratory specimens were used for RT-LAMP validation, it should be noted that the availability of freshly acquired specimen could result in even faster assay times, as would be the case when the assay is used as intended for clinical diagnosis.

4.5 The way forward

The development of this RSV RT-LAMP assay provides a foundation to explore several options of future research. The primary goal is the implementation of the RT-LAMP assay as a diagnostic test at the PoC in a resource-limited setting, followed by the possible commercialisation of the method. Such purpose requires a rigorous assessment of various key measures of success and usefulness of a diagnostic test for PoC. The reliability and accuracy of the RT-LAMP assay would have to be tested in the PoC setting and location for which it is intended, as performance charateristics of a PoC diagnostic test may differ in a clinical setting compared to a controlled laboratory environment.[122] To ascertain the applicable effect on patient-centred outcomes and ensure effective intervention, study in which RT-LAMP PoC testing is prospectively compared to laboratory testing could be a valuable indicator for PoC utility.[123]

The simplicity and isothermal nature of LAMP warrants the development of a low-tech thermal device conducive to RT-LAMP that can be used in low-resource settings, and which may be more practical for PoC application due to enhanced portability compared to a bulky water bath or even a heating block. For example, one study investigated the performance of non-instrumented nucleic acid (NINA) heaters for amplification of HIV-1 by RT-LAMP, based on the chemical exothermic reaction of calcium oxide in customised stainless-steel canisters.[124]

One challenge to rapid detection of RSV from a respiratory specimen using molecular assays is the necessity to first purify RNA, in order to remove RNAses and factors that could inhibit PCR. This process is time consuming and requires additional equipment and reagents. A simplified extraction method such as boiling has been used for DNA viruses and PCR has been successful from whole blood when the pH of buffers was increased in order to limit enzyme inhibition.[125] Therefore, exploration of simplified extraction methods that would precede RSV RT-LAMP could significantly enhance time-to-diagnosis for PoC testing.

Finally, to determine if RT-LAMP PoC testing would constitute good value, a comprehensive cost analysis encompassing all the above-mentioned factors should be done, based on an individual RT-LAMP diagnostic test at PoC.

4.6 Conclusions

RSV is an important contributor to the global health burden of ALRTI in the young and elderly. Early diagnosis of RSV infection is associated with shorter periods of hospitalisation and decreased mortality, necessitating rapid and sensitive PoC tests. This study described the development of a novel single-step multiplex RSV RT-LAMP assay with excellent sensitivity, specificity, and when combined with HNB dye could provide accurate visual diagnosis within 1 hour. Various methods and approaches were implemented throughout assay development and validation to improve ease of use, cost-efficiency and to streamline the end-to-end process for resource-limited use. The LAMP platform allows much scope for future development. We envisage that this multiplex RSV RT-LAMP assay will be used for rapid and sensitive RSV detection to challenge the current legacy of RSV diagnosis at the PoC.

Appendices:

Table A1. GenBank accession numbers of used RSV genomes.

Group	Accession number	Strain/Genotype
A	JX015498	GA2
	JQ901449	GA5
	JF920062	GA7
	AY911262	ATCC VR-26 (Long)
	M74568	A2
	U39662	S2
	NC_001803	RSS-2
	FJ948820	98-25147-X
	JX627336	GN435
	FJ614813	Line 19
B	AF013254	Wildtype B1
	AY353550	9320
	JN032116	B/WI/629
	JQ582843.1	NH1125

Protocol A1. Wizard SV clean-up of RT-LAMP amplification products.

Processing LAMP Amplifications:

1. Add an equal volume of Membrane Binding Solution to the LAMP amplification.

Binding of DNA:

2. Insert SV Minicolumn into Collection Tube.

3. Transfer prepared LAMP product to the Minicolumn assembly. Incubate at room temperature for 1 minute.

4. Centrifuge at 16,000 × *g* for 1 minute. Discard flow-through and reinsert Minicolumn into Collection Tube.

Washing:

5. Add 700 µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × *g* for 1 minute. Discard flow-through and reinsert Minicolumn into Collection Tube.

6. Repeat Step 4 with 500 µl Membrane Wash Solution. Centrifuge at 16,000 × *g* for 5 minutes.

7. Empty the Collection Tube and re-centrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution:

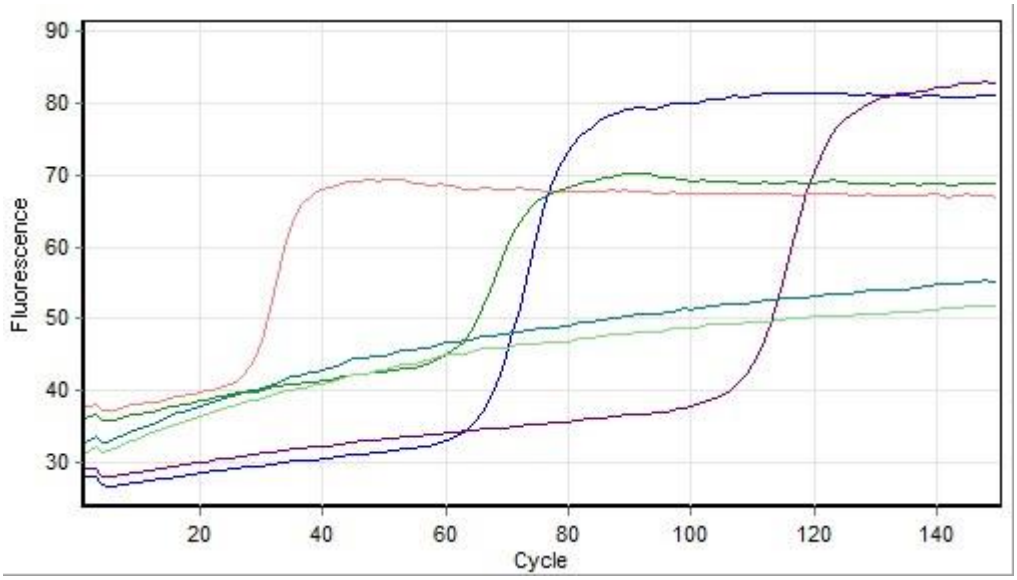
8. Carefully transfer Minicolumn to a clean 1.5 ml microcentrifuge tube.

9. Add 50 µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × *g* for 1 minute.

10. Discard Minicolumn and store DNA at 4°C or -20°C.

Appendix A1. Real-time reaction curves of RT-LAMP primer concentration optimisation.

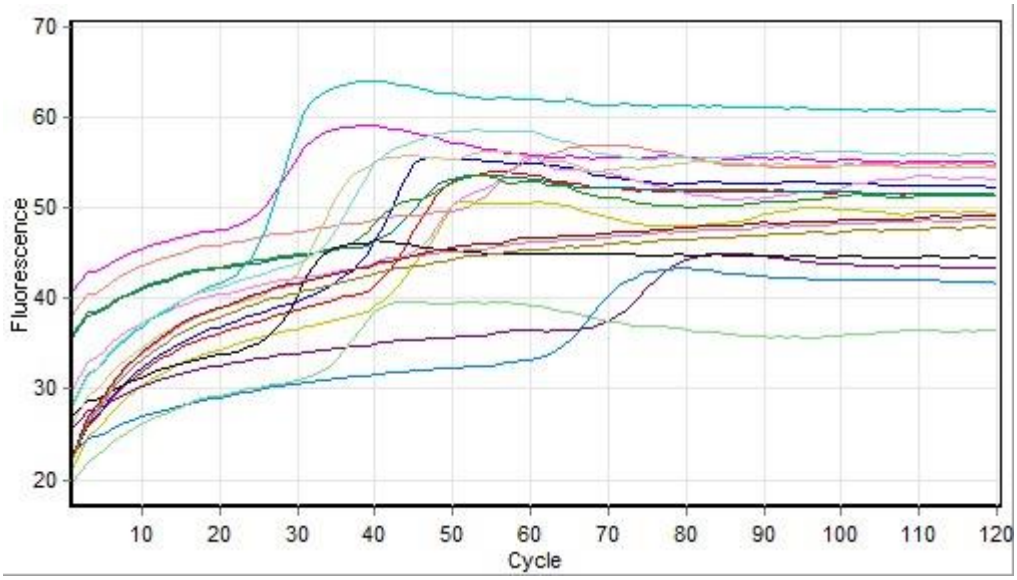
Raw Data For Cycling A.Green



No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		RSV-A2 (10^3)	Standard		1000
2		RSV-B2 (10^3)	Standard		1000
3		NTC2	NTC		
4		RSV-A1 (10^3)	Standard		1000
5		RSV-B1 (10^3)	Standard		1000
6		NTC1	NTC		

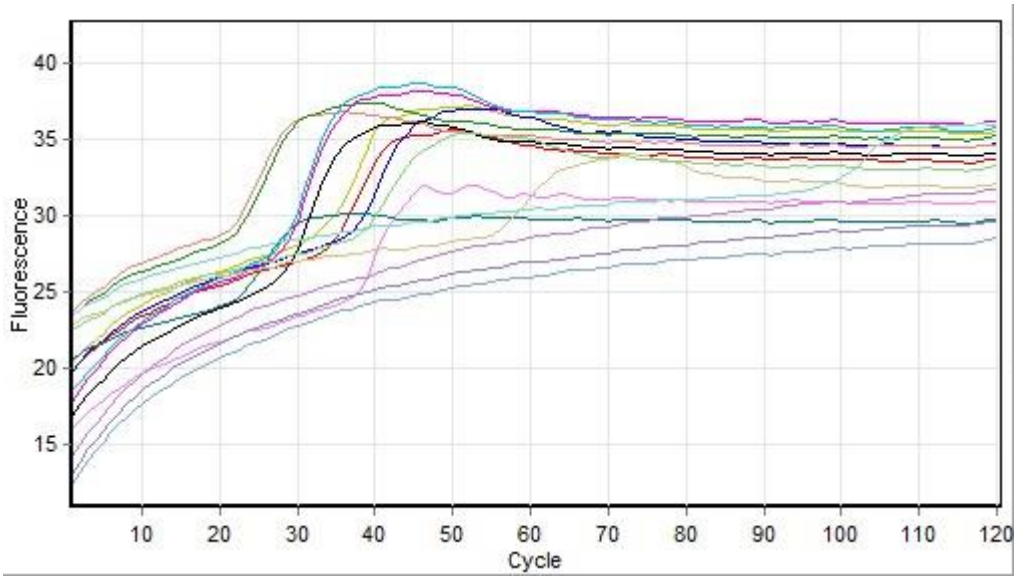
Appendix A2. Rotor-Gene Q curves for RSV positive specimens evaluated by RT-LAMP.

Raw Data For Cycling A.Green



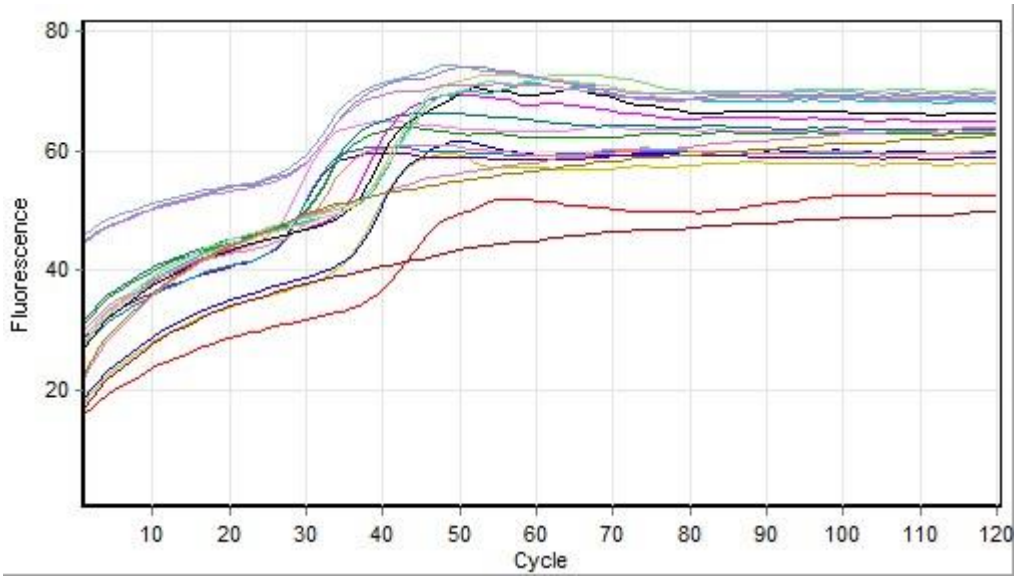
No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		A-Control	Standard		50
2		A-Control	Standard		50
3		A-Control	Standard		50
4		A1	Unknown		
5		A1	Unknown		
6		A1	Unknown		
7		A2	Unknown		
8		A2	Unknown		
9		A2	Unknown		
10		A3	Unknown		
11		A3	Unknown		
12		A3	Unknown		
13		A4	Unknown		
14		A4	Unknown		
15		A4	Unknown		
16		A5	Unknown		
17		A5	Unknown		
18		A5	Unknown		
19		NTC	NTC		
20		NTC	NTC		
21		NTC	NTC		

Raw Data For Cycling A.Green



No.	Colour	Name	Type	Ct
1		A6	Unknown	
2		A6	Unknown	
3		A6	Unknown	
4		A7	Unknown	
5		A7	Unknown	
6		A7	Unknown	
7		A8	Unknown	
8		A8	Unknown	
9		A8	Unknown	
10		A9	Unknown	
11		A9	Unknown	
12		A9	Unknown	
13		A10	Unknown	
14		A10	Unknown	
15		A10	Unknown	
16		NTC	NTC	
17		NTC	NTC	
18		NTC	NTC	

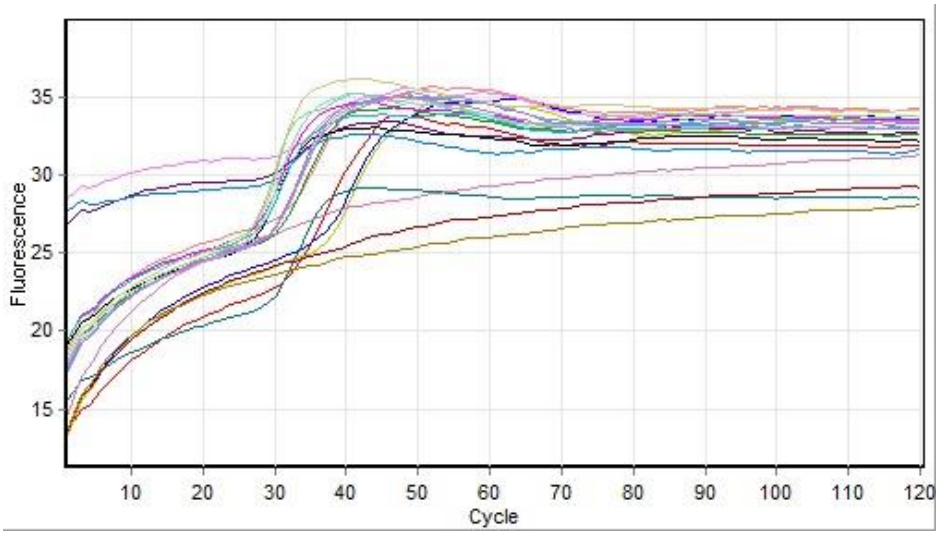
Raw Data For Cycling A.Green



No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		A-Control	Standard		50
2		A-Control	Standard		50
3		A-Control	Standard		50
4		A11	Unknown		
5		A11	Unknown		
6		A11	Unknown		
7		A12	Unknown		
8		A12	Unknown		
9		A12	Unknown		
10		A13	Unknown		
11		A13	Unknown		
12		A13	Unknown		
13		A14	Unknown		
14		A14	Unknown		
15		A14	Unknown		
16		A15	Unknown		
17		A15	Unknown		
18		A15	Unknown		
19		A16	Unknown		
20		A16	Unknown		
21		A16	Unknown		
22		NTC	NTC		
23		NTC	NTC		

No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
24		NTC	NTC		

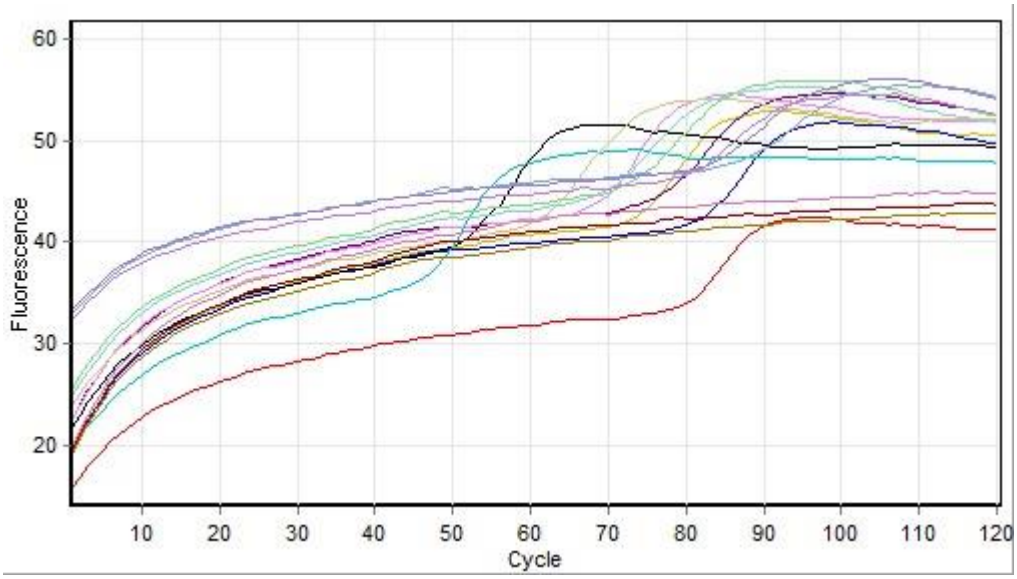
Raw Data For Cycling A.Green



No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		A-Control	Standard		50
2		A-Control	Standard		50
3		A-Control	Standard		50
4		A17	Unknown		
5		A17	Unknown		
6		A17	Unknown		
7		A18	Unknown		
8		A18	Unknown		
9		A18	Unknown		
10		A19	Unknown		
11		A19	Unknown		
12		A19	Unknown		
13		A20	Unknown		
14		A20	Unknown		
15		A20	Unknown		
16		A21	Unknown		
17		A21	Unknown		
18		A21	Unknown		
19		A22	Unknown		
20		A22	Unknown		
21		A22	Unknown		
22		NTC	NTC		

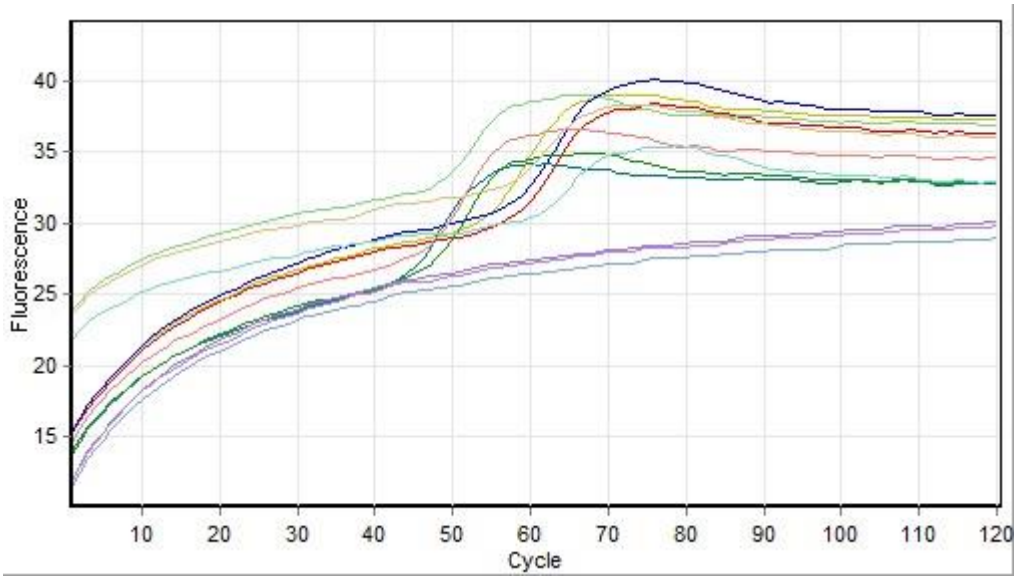
No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
23		NTC	NTC		
24		NTC	NTC		

Raw Data For Cycling A.Green



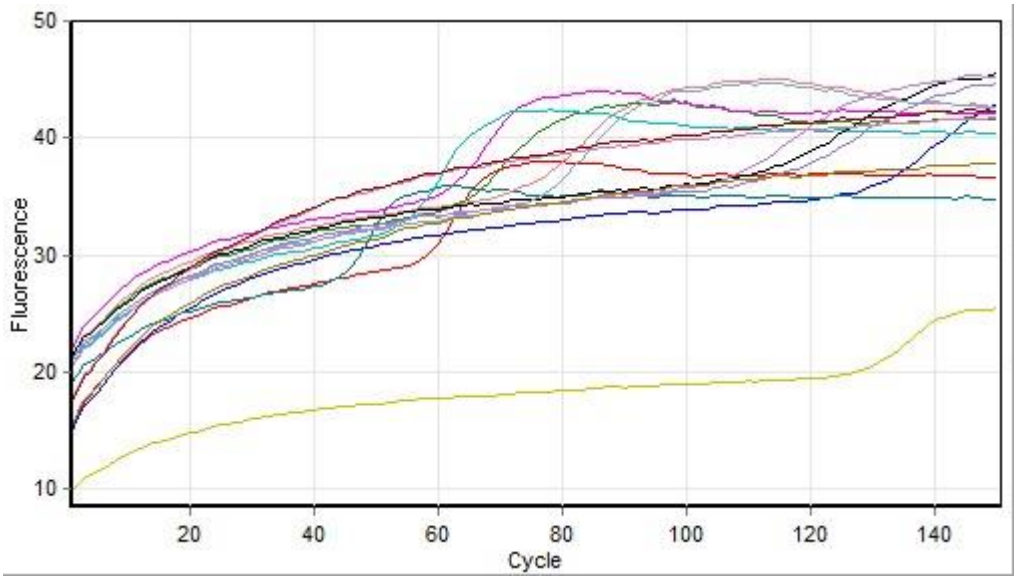
No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		B-Control	Standard		50
2		B-Control	Standard		50
3		B-Control	Standard		50
4		B1	Unknown		
5		B1	Unknown		
6		B1	Unknown		
7		B2	Unknown		
8		B2	Unknown		
9		B2	Unknown		
10		B3	Unknown		
11		B3	Unknown		
12		B3	Unknown		
13		B4	Unknown		
14		B4	Unknown		
15		B4	Unknown		
16		NTC	NTC		
17		NTC	NTC		
18		NTC	NTC		

Raw Data For Cycling A.Green



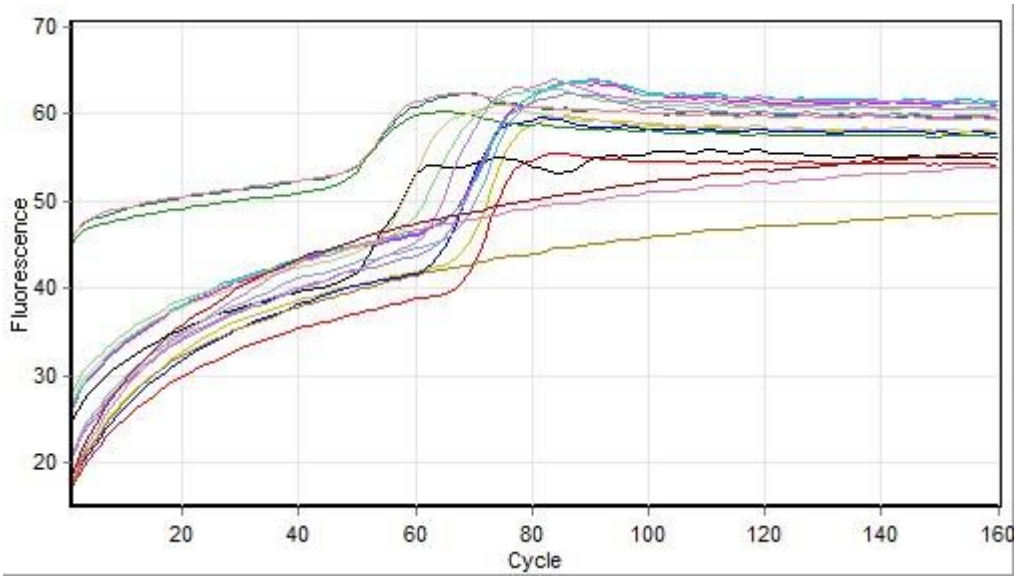
No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		B-Control	Standard		50
2		B-Control	Standard		50
3		B-Control	Standard		50
4		B5	Unknown		
5		B5	Unknown		
6		B5	Unknown		
7		B6	Unknown		
8		B6	Unknown		
9		B6	Unknown		
10		NTC	NTC		
11		NTC	NTC		
12		NTC	NTC		

Raw Data For Cycling A.Green



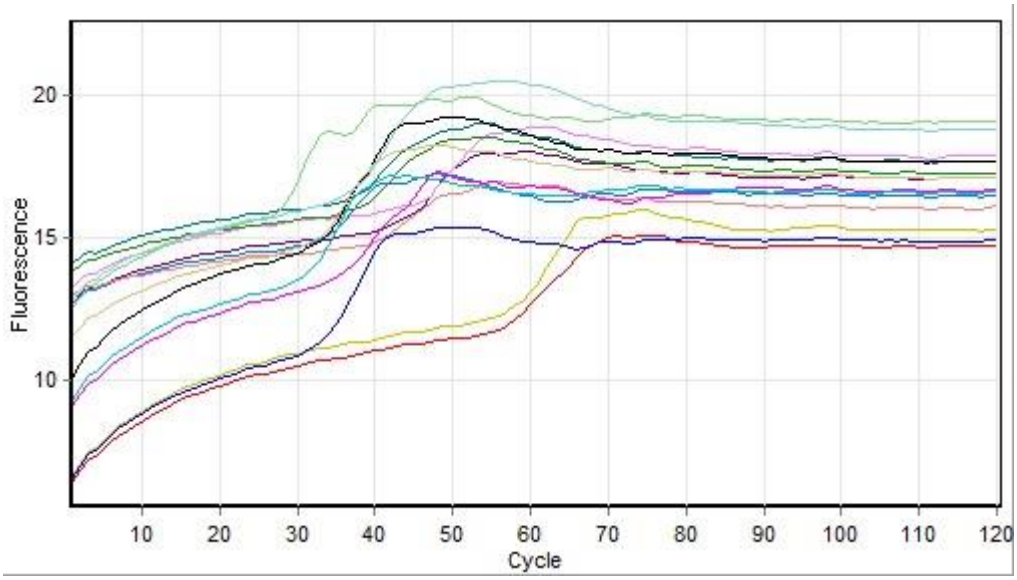
No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		B-Control	Standard		50
2		B-Control	Standard		50
3		B-Control	Standard		50
4		B7	Unknown		
5		B7	Unknown		
6		B7	Unknown		
7		B8	Unknown		
8		B8	Unknown		
9		B8	Unknown		
10		B9	Unknown		
11		B9	Unknown		
12		B9	Unknown		
13		NTC	NTC		
14		NTC	NTC		
15		NTC	NTC		

Raw Data For Cycling A.Green



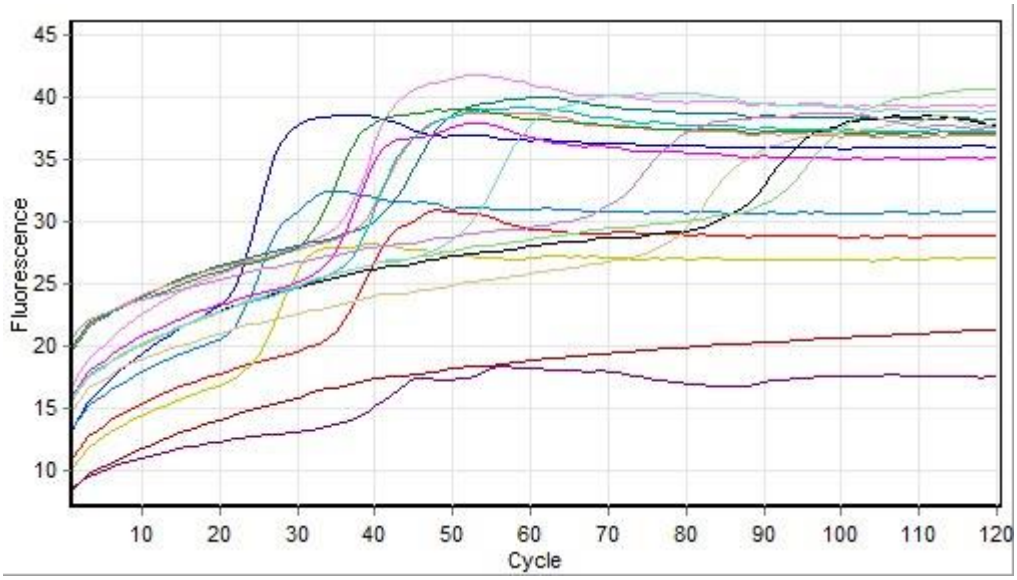
No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		B-Control	Standard		50
2		B-Control	Standard		50
3		B-Control	Standard		50
4		B10	Unknown		
5		B10	Unknown		
6		B10	Unknown		
7		B11	Unknown		
8		B11	Unknown		
9		B11	Unknown		
10		B12	Unknown		
11		B12	Unknown		
12		B12	Unknown		
13		B13	Unknown		
14		B13	Unknown		
15		B13	Unknown		
16		NTC	NTC		
17		NTC	NTC		
18		NTC	NTC		

Raw Data For Cycling A.Green



No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		B-Control	Standard		50
2		B-Control	Standard		50
3		B-Control	Standard		50
4		B14	Unknown		
5		B14	Unknown		
6		B14	Unknown		
7		B15	Unknown		
8		B15	Unknown		
9		B15	Unknown		
10		B16	Unknown		
11		B16	Unknown		
12		B16	Unknown		
13		B17	Unknown		
14		B17	Unknown		
15		B17	Unknown		
16		NTC	NTC		
17		NTC	NTC		
18		NTC	NTC		

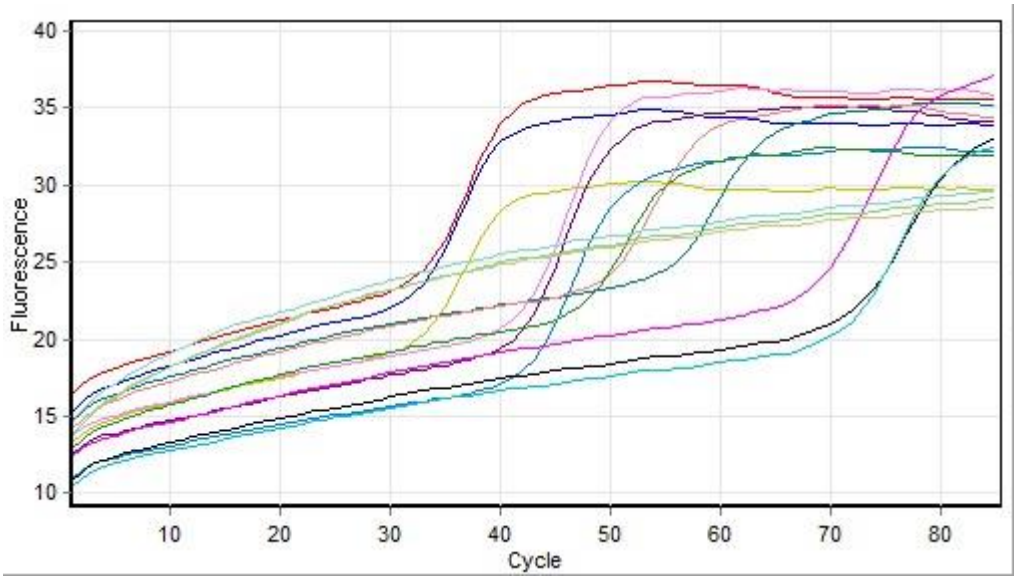
Raw Data For Cycling A.Green



No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		B-Control	Standard		50
2		B-Control	Standard		50
3		B-Control	Standard		50
4		B18	Unknown		
5		B18	Unknown		
6		B18	Unknown		
7		B19	Unknown		
8		B19	Unknown		
9		B19	Unknown		
10		B20	Unknown		
11		B20	Unknown		
12		B20	Unknown		
13		B21	Unknown		
14		B21	Unknown		
15		B21	Unknown		
16		B22	Unknown		
17		B22	Unknown		
18		B22	Unknown		
19		NTC	NTC		
20		NTC	NTC		
21		NTC	NTC		

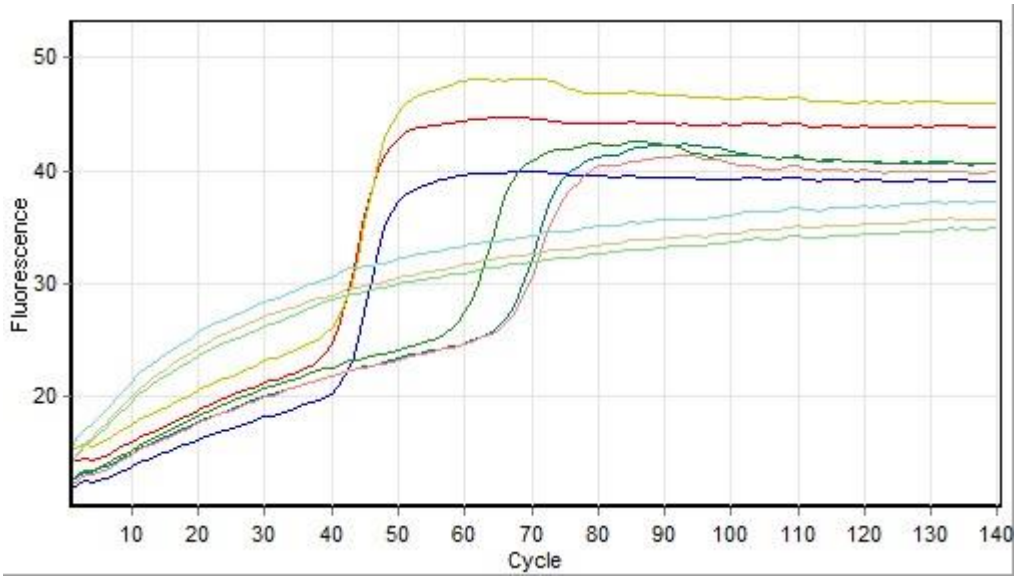
Appendix A3. Rotor-Gene Q curves for detection sensitivity of RT-LAMP RSV subgroups A and B.

Raw Data For Cycling A.Green



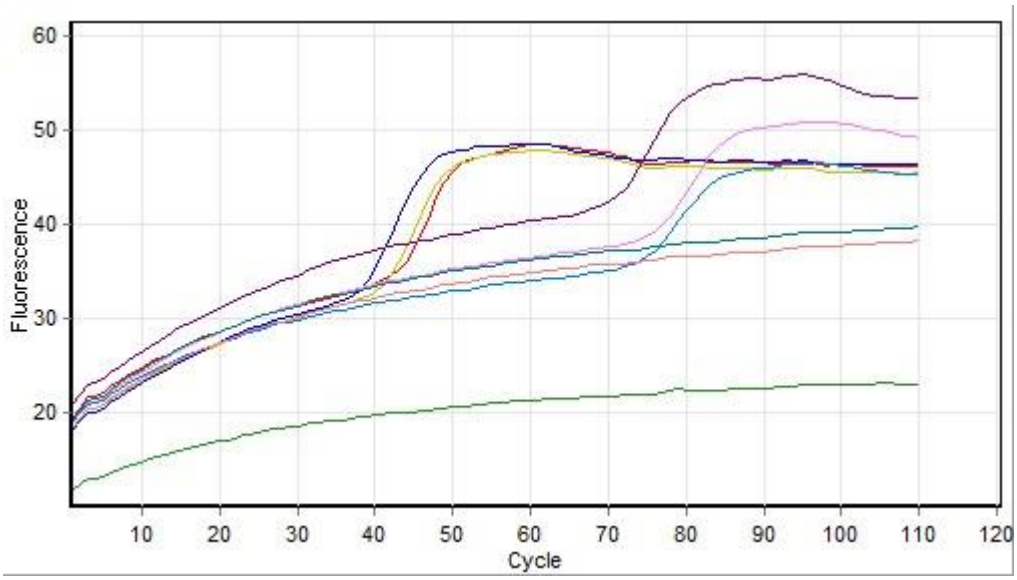
No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		RSV-A (10^4)	Standard		10000
2		RSV-A (10^4)	Standard		10000
3		RSV-A (10^4)	Standard		10000
4		RSV-A (10^3)	Standard		1000
5		RSV-A (10^3)	Standard		1000
6		RSV-A (10^3)	Standard		1000
7		RSV-B (10^4)	Standard		10000
8		RSV-B (10^4)	Standard		10000
9		RSV-B (10^4)	Standard		10000
10		RSV-B (10^3)	Standard		1000
11		RSV-B (10^3)	Standard		1000
12		RSV-B (10^3)	Standard		1000
13		NTC	NTC		
14		NTC	NTC		
15		NTC	NTC		

Raw Data For Cycling A.Green



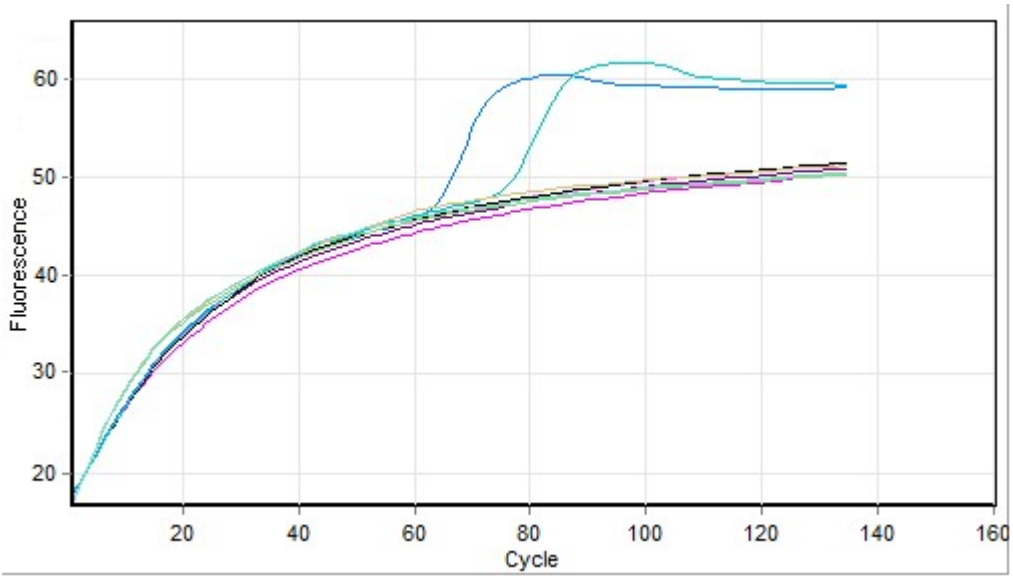
No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		RSV-A (10^2)	Standard		100
2		RSV-A (10^2)	Standard		100
3		RSV-A (10^2)	Standard		100
4		RSV-B (10^2)	Standard		100
5		RSV-B (10^2)	Standard		100
6		RSV-B (10^2)	Standard		100
7		NTC	NTC		
8		NTC	NTC		
9		NTC	NTC		

Raw Data For Cycling A.Green



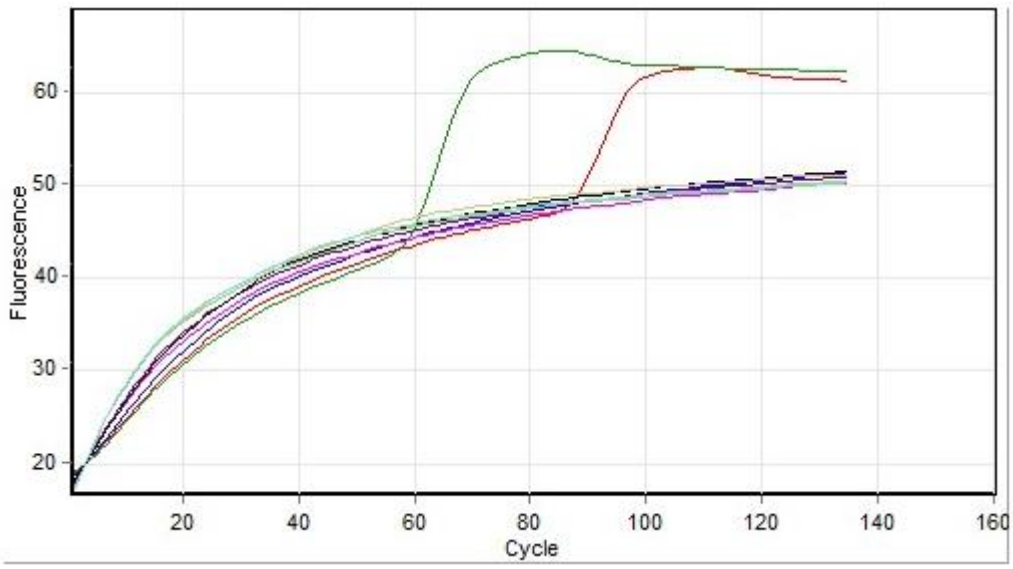
No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		RSV-A (10^1)	Standard		10
2		RSV-A (10^1)	Standard		10
3		RSV-A (10^1)	Standard		10
4		RSV-B (10^1)	Standard		10
5		RSV-B (10^1)	Standard		10
6		RSV-B (10^1)	Standard		10
7		NTC	NTC		
8		NTC	NTC		
9		NTC	NTC		

Raw Data For Cycling A.Green (5 FFU's Run 1)



No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		RSV-A (10^0.5)	Standard		5
2		RSV-A (10^0.5)	Standard		5
3		RSV-A (10^0.5)	Standard		5
4		RSV-B (10^0.5)	Standard		5
5		RSV-B (10^0.5)	Standard		5
6		RSV-B (10^0.5)	Standard		5
7		NTC	NTC		
8		NTC	NTC		
9		NTC	NTC		

Raw Data For Cycling A.Green (5 FFU's Run 2)



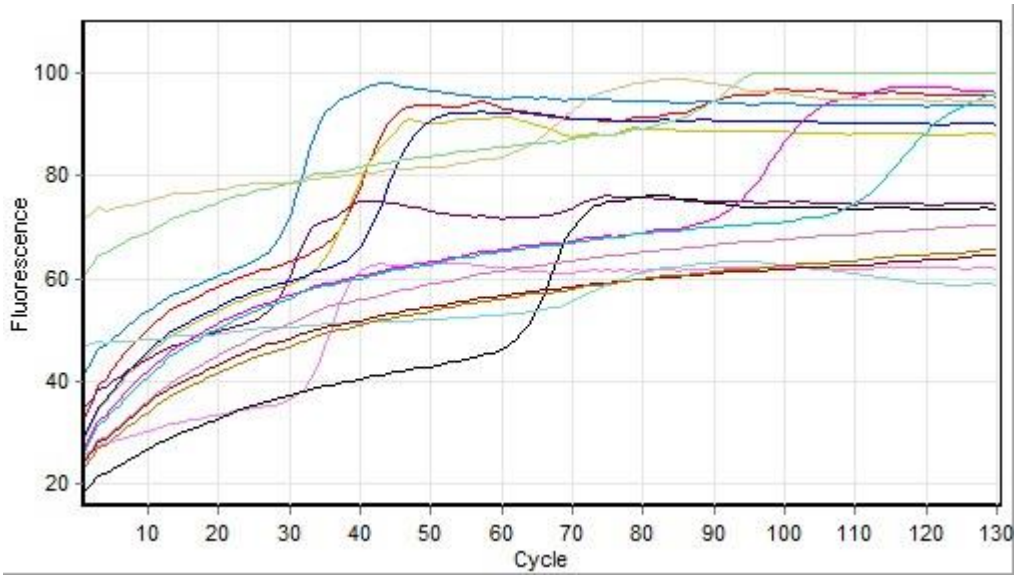
No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		RSV-A (10^5)	Standard		5
2		RSV-A (10^5)	Standard		5
3		RSV-A (10^0.5)	Standard		5
4		RSV-B (10^5)	Standard		5
5		RSV-B (10^0.5)	Standard		5
6		RSV-B (10^0.5)	Standard		5
7		NTC	NTC		
8		NTC	NTC		
9		NTC	NTC		

Table A3. Amplification times for RT-LAMP sensitivity testing for RSV A and B.

Specimen FFU/reaction	Amplification Time (min:sec)	Specimen FFU/reaction	Amplification Time (min:sec)
A10^4	16:00	B10^4	27:30
A10^4	16:00	B10^4	25:00
A10^4	17:00	B10^4	23:00
A10^3	21:00	B10^3	33:00
A10^3	21:00	B10^3	35:00
A10^3	21:00	B10^3	35:00
A10^2	21:30	B10^2	30:00
A10^2	21:30	B10^2	34:00
A10^2	22:00	B10^2	34:00
A10^1	20:00	B10^1	36:30
A10^1	21:30	B10^1	39:00
A10^1	21:30	B10^1	39:00
A10^0.5	no amplification	B10^0.5	no amplification
A10^0.5	no amplification	B10^0.5	no amplification
A10^0.5	34:00	B10^0.5	38:00
A10^0.5	43:00	B10^0.5	31:00
A10^0.5	no amplification	B10^0.5	no amplification
A10^0.5	no amplification	B10^0.5	no amplification

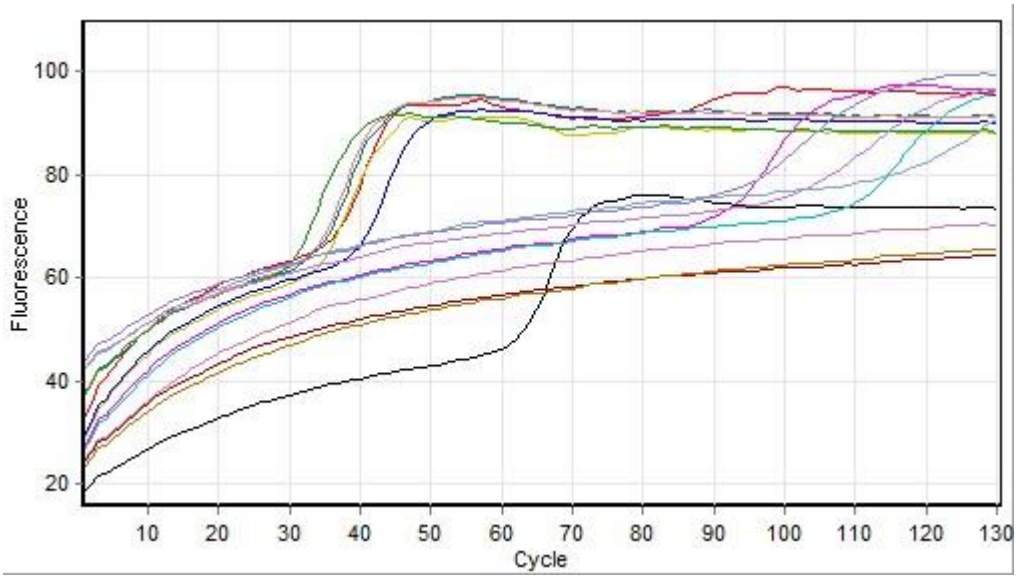
Appendix A4. Rotor-Gene Q curves for reproducibility of RT-LAMP testing for RSV A and B.

Raw Data For Cycling A.Green



No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		A-Control	Standard		50
2		A-Control	Standard		50
3		A-Control	Standard		50
4		A1	Unknown		
5		A2	Unknown		
6		A3	Unknown		
7		B-Control	Standard		50
8		B-Control	Standard		50
9		B-Control	Standard		50
10		B1	Unknown		
11		B2	Unknown		
12		B3	Unknown		
13		NTC	NTC		
14		NTC	NTC		
15		NTC	NTC		

Raw Data For Cycling A.Green



No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
1		A-Control	Standard		50
2		A-Control	Standard		50
3		A-Control	Standard		50
4		A1	Unknown		
5		A2	Unknown		
6		A3	Unknown		

No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)
7		B-Control	Standard		50
8		B-Control	Standard		50
9		B-Control	Standard		50
10		B1	Unknown		
11		B2	Unknown		
12		B3	Unknown		
13		NTC	NTC		
14		NTC	NTC		
15		NTC	NTC		

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