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Original Article

A quite sensitive fluorescent loop-mediated isothermal amplification for rapid detection of respiratory syncytial virus

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Abstract

Introduction: Human respiratory syncytial virus (hRSV) is a common respiratory virus closely related to respiratory tract infection (RTI). Rapid and accurate detection of hRSV is urgently needed to reduce the high morbidity and mortality due to hRSV infection.

Methodology: Here, we established a highly sensitive and specific reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay for the rapid detection of A and B group hRSV simultaneously. The specific primer sets for hRSV A and B groups were designed in the M and M2-2 gene, respectively. SYTO 9 was used as the fluorescent dye for real-time monitoring of the amplification of hRSV RNA without cross reaction between hRSV A and B.

Results: The limit of detection (LOD) of our new method was 281.17 50% tissue culture infective doses (TCID50)/mL for hRSV A and 1.58 TCID50/mL for hRSV B. Using 90 clinical samples, a comparison to traditional RT-PCR was performed to validate this assay. The positivity rate of RT-LAMP and RT-PCR were 67.8% and 55.6%, respectively, and the positivity rate of RT-LAMP was significantly higher than RT-PCR (χ^2 test, $P < 0.01$).

Conclusions: Compared with traditional RT-PCR method, the newly developed fluorescent RT-LAMP combined with well-designed primers and SYTO 9 is quite sensitive, specific, rapid and well applicable to hRSV clinical diagnosis.

Key words: Reverse-transcription loop-mediated isothermal amplification; respiratory syncytial virus; SYTO 9; sensitivity; specificity; clinical diagnosis.

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Introduction

From 1960s, a series of epidemiological investigation based on virus isolation found that upper respiratory tract infection (URTI) and severe lower respiratory tract infection (LRTI) including bronchitis and pneumonia were mainly caused by hRSV [1–5]. Prevalent all over the world, hRSV is a highly infectious respiratory pathogen that can repeatedly infect a patient for many times throughout life. The proportion of hospitalization in hRSV infected patients was about 1~5% [6] with a fatality rate of 1~3% [7] . The main susceptible population of hRSV are infants and young children. Nearly all children under 2 years old worldwide are infected with hRSV at least once. About 47% and 45% of children with infection under 1 year old are infected again in the second and third year, respectively [8].

LRTI is caused by multiple pathogens probably comprised of bacteria, viruses and mycoplasma, among others [9,10]. However, in mainland China, URTI or LRTI patients with negative C reactive protein are typically treated with antiviral drugs in combination with antibiotics [11,12]. Although URTI or LRTI in children could be well-controlled by doctors, patients might be excessively or incorrectly treated without clear diagnosis of clinical pathogen [13,14]. Because of the lack of diagnostic tools, and the shortage of effective vaccine and anti-viral drugs, hRSV infections lead to high morbidity and mortality among pre-school children [15–18]. Thus, a rapid and accurate detection of hRSV from clinical samples is extremely necessary for the effective treatment of RTI.

There are more than 15 commercially available RSV diagnosis assays developed in last decade [15].

Those assays are based on different mechanisms including, but not limited to culture, antigen-antibody interaction, nucleic acid amplification and oligo-nucleotide hybridization [19,20]. Antigen-based methods such as Immunofluorescent rapid diagnosis assay (IF) and Alkaline Phosphatase-Anti-Alkaline Phosphatase technique (APAAP) are widely used and quite sensitive [21,22]. Nucleic acid amplification tests (NATs) are PCR-based assays. Most commercially available NATs are normal multiplex PCR/RT-PCR or corresponding qPCR based strategies [23,24].

In recent years, fully developed commercial qPCR kits are easy-to-use and predominant with high specificity, sensitivity, reproducibility and low risks of contamination [25]. However, qPCR is rather expensive, especially the expenses for probe synthesis and fluorescent thermal cycler to preserve two or more temperatures in the whole program [26]. Considering the disadvantage of qPCR, loop-mediated isothermal amplification (LAMP) with a high sensitivity and specificity was invented in 2000 [27]. Performed under isothermal conditions, it is much simpler and cheaper than qPCR [27–30]. In those years, several improvements were added to LAMP to develop different methods including RT-LAMP, six-primer (loop primer) method and real-time quantitative LAMP (Q-LAMP) [30]. Q-LAMP is now a promising technology which can detect pathogens accurately from the environment [31].

In our study, an efficient RT-LAMP assay was developed with more conserved primer pairs and SYTO 9 as the real-time florescent dye to specifically detect A and B groups of hRSV. The LOD of the novel method was 281.17 TCID50/mL for hRSV A and 1.58 TCID50/mL for hRSV B. The comparison showed that the florescent RT-LAMP had higher positivity rate for RSV detection than traditional RT-PCR. Our results indicate that this prominent new RT-LAMP is quite sensitive, specific, rapid and well applicable to hRSV clinical diagnosis in economic less-developed areas.

Methodology

Virus strains and clinical samples

Standard virus strains including hRSV group A (VR-1540, $5\times10^{5.75}$ TCID50/mL)/group B (VR-1400, $5\times10^{4.5}$ TCID50/mL) and all the other 8 reference virus strains, which were purchased from ATCC (Manassas, VA, USA) and cultured according to ATCC recommended conditions, were used in this study. The outpatient children aged between 2 months and 12 years, mainly from 1 to 6 years, diagnosed as tonsillitis, bronchitis, upper respiratory tract infections and pneumonia with running rose, cough, fever, headache, muscular soreness, sore throat, expectoration, rales, rash, stomachache, dyspnea and chest pain were included in this study. A total of 90 nasopharyngeal swabs from those patients between 2009 and 2012 in Shanghai Nanxiang Hospital were obtained. Briefly, nasopharyngeal swabs were immersed in virus transport media containing Hank's buffer, bovine serum albumin (BSA), hydroxyethyl piperazine ethanesulfonic acid (HEPES) and antibiotics. The supernatant was mixed, aliquoted into three tubes and stored at −80°C. The samples were obtained from previous two studies with informed consent from each child's parent or guardian [32,33]. The study was approved by the Ethics Committee of the Shanghai Public Health Clinical Center.

Nucleic acid extraction

RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Venlo, Netherlands) by Wang's method [34]. In short, RNA was extracted from 140 µl of standard virus strains and clinical specimens and eluted in 40 µL Nuclease-free H_2O according to the manufacturer's instructions. The extracted RNA was diluted at different concentrations and stored at -80°C for subsequent use.

RT-LAMP primer design

The most conserved regions of hRSV A and B groups were determined according to the alignments of 49 RSV genomic sequences. The LAMP primers for RSV A/B groups, which respectively located in the M and M2-2 gene were designed using Primer Explorer (http://primerexplorer.jp/lampv5e/index.html) and modified to avoid variable sites (Supplementary Figure 1). The detailed primer information is shown in Supplementary Table 1.

Optimization of the fluorescent RT-LAMP assay for rapid hRSV detection

The reaction system was established according to the RT-LAMP reaction mix used in Mu (2014) [35], and then optimized by the orthogonal experiments. Briefly, the RT-LAMP was performed with primers, dNTPs, WarmStart RTx Reverse Transcriptase (NEB, MA, USA) and Bst 2.0 WarmStart DNA Polymerase (NEB, MA, Ipswich, USA) combinations in isothermal buffer (10X) with 0.4 µM SYTO 9 (Life technologies, CA, Carlsbad, USA) as the fluorescent dye. Twenty five µl reaction including 5 µl template input was run on a LC96 Real-Time PCR System (Roche, Baden, Grenzach-Wyhlen, Germany). Reaction condition was

Table 1. Reaction condition sets designed from the orthogonal table.

Number	Tween20 %	dNTP mM	Betaine M	Mg^{2+} mM
1	0	1.0	0	10
2	0.1	1.4	0.4	10
3	0.3	1.6	0.8	10
4	0.3	1.4	0	8
5	0	1.6	0.4	8
6	0.1	1.0	0.8	8
7	0.1	1.6	0	6
8	0.3	1.0	0.4	6
9	0	1.4	0.8	6

60 cycles of 62°C for 60 seconds. Then the betaine concentrations were further optimized and the final reaction mix was determined.

Specificity and sensitivity of the novel fluorescent hRSV RT-LAMP assay

The specificity of the fluorescent RSV RT-LAMP was assessed using 10 common respiratory viruses including hRSV A and B groups, PIV-3, EV68, HRV, Adv, 229E, Flu A, Flu B, and Oc43. The LOD of our new assay was determined by Mu's (2014) [35] method. Serially diluted RNA stocks from human RSV group A ($5\times10^{5.75}$ TCID50/mL) and group B ($5\times10^{4.5}$ TCID50/mL) were used to determine the detection limit of the assay. The TCID50 of VR-1400 and VR-1540 were determined using the Reed-Muench method in HEP-2 and Vero cells, respectively.

Comparison between the sensitive fluorescent RT-LAMP and traditional RT-PCR

To evaluate the sensitive fluorescent RT-LAMP for hRSV detection, a traditional one-step RT-PCR described by Zhang [36] was used to validate the sensitive fluorescent RT-LAMP using 90 RTI clinical samples. In brief, primer GA480 or GB496 (400 nM) was mixed with 400 nM of primer F164, 1 μL dNTP mix (containing 10 mM of each dNTP), 5 μL of 5X Qiagen OneStep RT-PCR buffer, 1 μL of enzyme mix, 2.5 μL of extracted RNA and 13.5 μL of RNase-free water. The amplification process was initiated with a reverse transcription step at 50°C for 30 minutes, followed by denaturation at 95°C for 15 minutes, 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, with a final extension of 72°C for 10 minutes. The products were detected by electrophoresis in 1.5% agarose gel stained with GelRed (Biotium, CA, Hayward, USA). Both the results of RT-PCR and the sensitive fluorescent RT-LAMP were collected, compared and analysed.

Results

Establishment of a fluorescent RT-LAMP assay for rapid RSV detection

The new fluorescent RT-LAMP reaction system was established referring to our published method [35] with best primer sets located in the more conserved region, and then optimized with the orthogonal experiments [37]. Briefly, experiments were designed according to four factors and three different levels (Supplementary Table 2) orthogonal table (Table 1), and optimized by RT-LAMP assay with SYTO 9 as the fluorescent dye. The RNA templates of RSV groups A and B with 1:100 dilutions were used in the new fluorescent RSV RT-LAMP assay. The reactions were carried out, then the amplification curves were plotted as in Figure 1A. It is very clear that reaction condition 5 and 9 were more efficient than other conditions, which reached the leveling off stage in 15 minutes.

Based on the optimal reaction mix obtained by the orthogonal experiments (Figure 1A), we further optimized the reaction mix by changing the final concentrations of betaine at 0/0.2/0.4/0.6/0.8M (data not shown). The best reaction mix was 1.4mM dNTP and 6mM Mg^{2+} without Tween20 and betaine. With this reaction condition used in subsequent experiments, the results showed that the new primer sets had high amplification efficiency regardless of hRSV groups A and B (Figure 1B), indicating that the new primer sets were suitable for hRSV rapid detection.

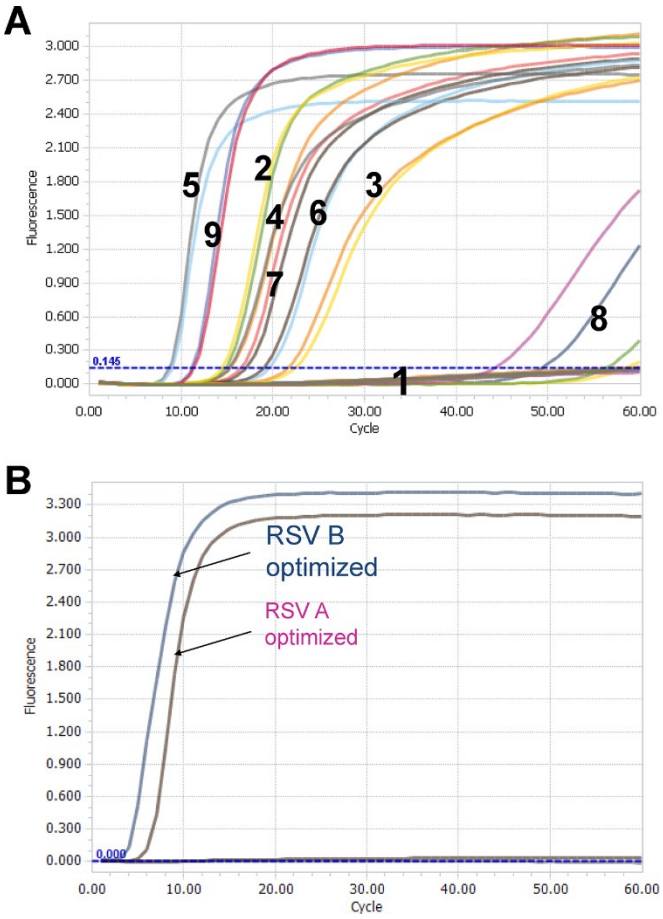
Specificity and sensitivity of the fluorescent RSV RT-LAMP assay

The specificity of the fluorescent RT-LAMP was assessed by testing with nucleic acid extracted from ten standard respiratory virus strains as mentioned previously in a 0.4μM SYTO 9 reaction mix. The results showed that well amplification occurred in the reactions with nucleic acids of hRSV A and B, but did not occur in the reaction with nucleic acids from other viruses (Figure 2A and 2B). Furthermore, we found that the primer set for hRSV A group only amplify group A

and the primer set for hRSV B group only amplify group B, indicating that there is no cross reaction between groups A and B primer sets (Figure 2C).

The RNA of hRSV A (VR-1540, $5\times10^{5.75}$ TCID50/mL) and hRSV B (VR-1400, $5\times10^{4.5}$ TCID50/mL) was serially 10-fold diluted as templates to analyze the LOD of the new fluorescent hRSV RT-LAMP. Relative fluorescent amplification curves were recorded. The results showed that the LOD for hRSV groups A and B were $5\times10^{1.75}$ TCID50/mL (Figure 3A) and $5\times10^{-0.5}$ TCID50/mL (Figure 3B), respectively. Both of the LOD of hRSV A and B were lower than the clinical sample viral loads [38]. The new fluorescent RT-LAMP assay for group B had a lower LOD. The amplification of B group primers was more efficient than A group primers (Figure 3A and 3B).

Figure 1. Optimization of the RT-LAMP assay with SYTO 9 for rapid RSV detection.

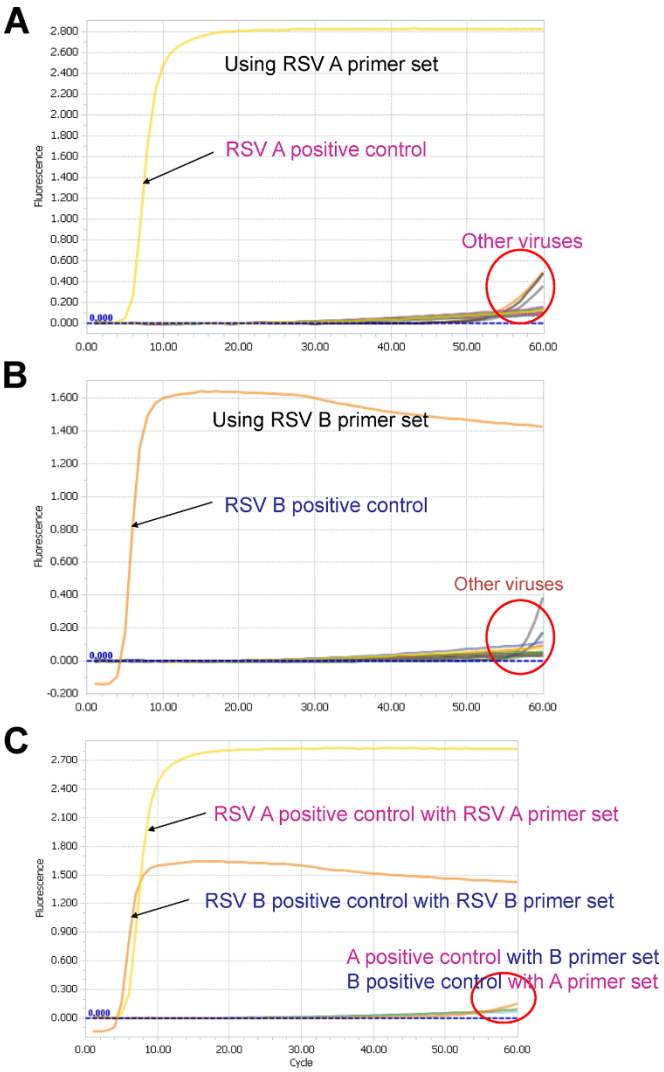


(A) shows the orthogonal experiments to find the optimal reaction mix. 1-9 represents the optimized reaction mix described and listed in Table 1. Condition 5 and 9 are better for hRSV amplification. (B) the performance of RSV RT-LAMP with SYTO 9 and newly designed primers. The reaction with newly designed primers had very rapid amplification of hRSV A and B. The experiments were repeated at least 3 times with 2 duplicates each time. The representative results are shown.

Evaluation of the sensitive fluorescent RT-LAMP using clinical samples

To validate the RT-LAMP assay for hRSV detection, we compared the sensitive fluorescent RT-LAMP with traditional RT-PCR method using 90 clinical samples from nasopharyngeal swabs collected from children with RTIs in Nanxiang Hospital, Shanghai, China. The positivity rate of RT-LAMP and RT-PCR were 67.8% and 55.6%, respectively. The positivity rate by RT-LAMP was significantly higher than that by RT-PCR (χ^2 test, $P < 0.01$) (Table 2). In particular, there were 17 samples that were detected as positive by RT-LAMP, but as negative by RT-PCR. In

Figure 2. Specificity of the new fluorescent RT-LAMP assay tested using ten common respiratory viruses.



(A) the reaction with group A primer set; (B) the reaction with group B primer set; (C) the cross-reaction experiment using group A primers to amplify group B viruses and using group B primers to amplify group A viruses. The experiments were repeated at least 3 times with 2 duplicates each time. The representative results are shown.

Table 2. Comparison of RT-LAMP with RT-PCR in the detection of 90 clinical samples.

N=90	RT-PCR positive	RT-PCR negative	Total	RT-PCR positive rate
RT-LAMP positive	44	17	61	
RT-LAMP negative	6	23	29	
Total	50	40	90	55.6%
RT-LAMP Positive rate			67.8%	

contrast, there were only 6 positive samples by RT-PCR that were detected as negative by RT-LAMP (Table 2). These results suggest that the new fluorescent RT-LAMP has higher detection sensitivity than traditional RT-PCR.

Discussion

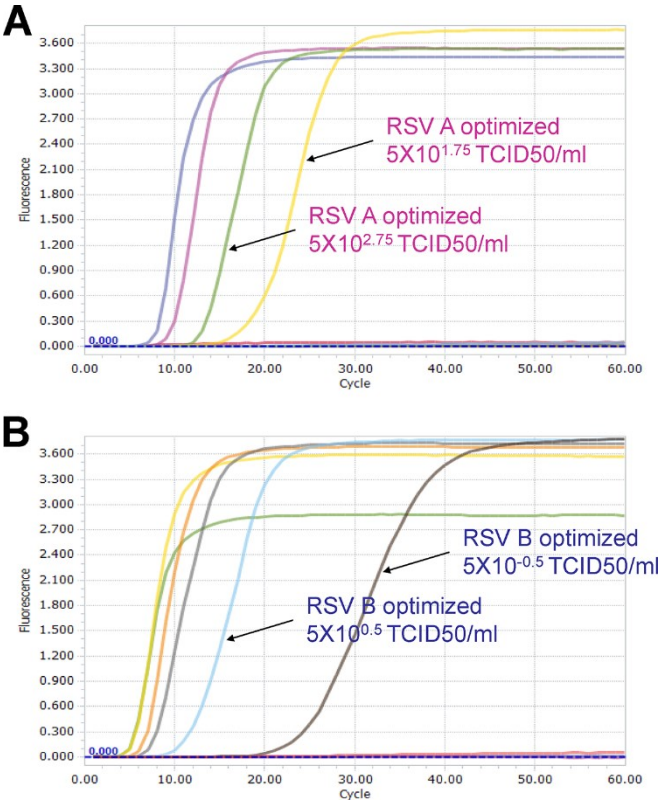
LAMP is a DNA amplification technique with advantages over traditional PCR [27]. Over the past decades, it was further developed in combination with other molecular methods, including reverse transcription and multiplex amplification [39,40]. Currently, the main varieties of LAMP assay available are designed by colorimetric change [41,42], real-time monitored [39] with fluorescent dye or using turbidity meter [43]. Nowadays, LAMP is widely applied as new diagnostics for infectious diseases. Lyophilized LAMP reagents kit [44] and LAMP primer sets are already available for detection of pathogenic microorganisms [43].

Many LAMP reaction results could be visualized by adding DNA Chimeric dyes such as ethidium bromide, SYBR Green I, propidium iodide and Calcein after incubation for endpoint analysis [45]. But those open-cap methods lead to the increment of amplicon contamination. In this study, a new sensitive fluorescent hRSV RT-LAMP assay with SYTO 9 was developed. This assay is the first method using SYTO 9, a more adaptable fluorescent dye than Calcein, to real-time monitor the hRSV RT-LAMP reaction. It could avoid possible contamination, is more convenient to operate and resulting in more sensitive results. Our results suggest that SYTO 9 might be widely used in real-time monitored LAMP reaction and added before incubation as an easy-to-use assay.

Compared with traditional RT-PCR method and our previous RT-LAMP with Calcein, we proved that the RT-LAMP assay with SYTO 9 and new primer sets had high sensitivity and specificity to detect hRSV. For RSV detection using clinical samples, the positivity rate by this new RT-LAMP with SYTO 9 is much higher than that by RT-PCR, while the positivity rate by our previous RT-LAMP with Calcein is equal to RT-PCR [35]. This may due to the better primer sets and the substitution of Calcein by SYTO 9. As previously reported, used as an in-tube indicator, Calcein may decrease the sensitivity of LAMP for real-time amplification monitoring [46].

As on-going approaches used in laboratory monitoring of RSV, the costs of Alere BinaxNow RSV Card and the traditional RT-PCR are about $ 8 and $ 6 per reaction, respectively. Our newly developed RT-LAMP with SYTO 9 costs only $ 2 per reaction, is 3~4 times cheaper than traditional RT-PCR and rapid antigen detection tests (RADT). The specific hRSV amplification could be finished within 50 minutes, which is about 30-60 minutes shorter than the

Figure 3. Sensitivity of the assay tested using serially diluted RNA of hRSV standard strains.



(A) Amplification curve of RT-LAMP detected by hRSV group A primer set. The extracted nucleic acid was diluted from $5\times10^{5.75}$ TCID50/mL to $5\times10^{-0.25}$ TCID50/mL; (B) Amplification curve of RT-LAMP detected by hRSV group A primer set. The extracted nucleic acid was diluted from $5\times10^{4.5}$ TCID50/mL to $5\times10^{-1.5}$ TCID50/mL. The experiments were repeated at least 3 times with 2 duplicates each time. The representative results are shown.

traditional RT-PCR. Furthermore, the new fluorescent RT-LAMP with SYTO 9 is easy-to-handle, and does not need very skilled staff. The results are readable with more accuracy than traditional RT-PCR needing agarose electrophoresis. In particular, the new hRSV RT-LAMP assay can be easily updated and developed into a visual measurement qualitative assay by adding a minuscule amount of high-fidelity DNA polymerase and colorimetric dyes (e.g. HNB and cresol red) for board-spectrum detection of various RSV variants[47,48]. So, the new RT-LAMP represents a fast, sensitive and accurate real-time detection method, and has great potentials to be developed into a promising point-of-care testing (POCT) assay in developing countries with limited resources.

Conclusion

Shortly, we exploited a new RT-LAMP assay to detect hRSV combined with SYTO 9. The new fluorescent RT-LAMP assay has high sensitivity and specificity, and can finish the target amplification reaction quickly under 62°C. Monitored by real-time PCR instruments, it is comprehensively applicable in clinical diagnosis for rapid hRSV detection economically, and will provide precise evidence for the treatment of hRSV caused RTI.

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Annex – Supplementary Items

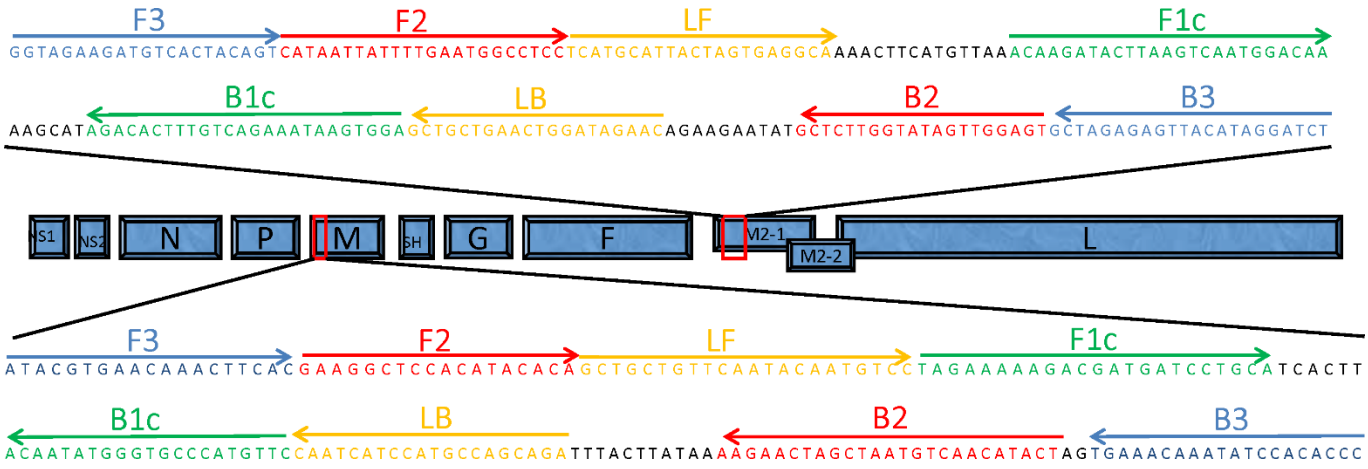
Supplementary Table 1. The primer information for the detection of hRSV group A and B.

Genotypes	LAMP primers	Primer sequences (5'-3')
Group A	F3	ATACGTGAACAAGCTTCAC
	B3	GGGTGTGGATATTTGTTTCA
	FIP	TGCAGGGTCATCGTCTTTTTCTA-GAAGGCTCCACATACACA
	BIP	ACAATATGGGTGCCCATGTTC-AGTATGTTGACATTAGCTAGTTCTT
	LF	GGACATTGTATTGAACAGCAGC
	LB	CAATCATCCATGCCAGCAGA
Group B	F3	GGTAGAAGATGTCACTACAGT
	B3	AGATCCTATGTAACTCTCTAGC
	FIP	TTGTCCATTGACTTGAGTATCTTGT-CATAATTACTTTGAATGGCCTCC
	BIP	AGACACTTTGTCTGAAATAAGTGGA-ACTCCAACTATACCAAGAGC
	LF	TGCCTCACTAGTAAGGCATGA
	LB	GCTGCTGAACTGGACAGAAC

Supplementary Table 2. Condition levels of each factor under optimization (final concentration).

Level	Tween20 %	dNTP mM	Betaine M	Mg^{2+} mM
1	0	1.0	0	6
2	0.1	1.4	0.4	8
3	0.3	1.6	0.8	10

Supplementary Figure 1. The genomic location of LAMP primers for hRSV detection.



The genomic location of LAMP primers for hRSV detection. RT-LAMP primers for hRSV including F3 (forward outer primer), F2 and F1c (forward inner primers), B2 and B1c (reverse inner primers), B3 (reverse outer primers), LF and LB (two loop primers). The genome of hRSV group A and B, including NS1, NS2, N, P, M, SH, G, F, M2-1, and M2-2 genes. Non-structural protein (NS).