



## Virology

## A highly sensitive 1-tube nested real-time RT-PCR assay using LNA-modified primers for detection of respiratory syncytial virus



Li Zhao<sup>a,b,1</sup>, Ji Wang<sup>b,1</sup>, Gui-xia Li<sup>c,1</sup>, Fang-zhou Qiu<sup>a,b</sup>, Chen Chen<sup>b</sup>, Meng-chuan Zhao<sup>c</sup>, Le Wang<sup>c</sup>, Su-xia Duan<sup>b,c</sup>, Zhi-shan Feng<sup>c,\*</sup>, Xue-jun Ma<sup>b,\*\*</sup>

<sup>a</sup> Hebei Medical University, Shijiazhuang, 050031, Hebei, China

<sup>b</sup> Key Laboratory for Medical Virology, National Health and Family Planning Commission, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, 102206, China

<sup>c</sup> Children's Hospital of Hebei Province, Shijiazhuang, 050031, Hebei, China

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## ABSTRACT

Respiratory syncytial virus (RSV) causes serious respiratory tract infection worldwide. The relatively low RSV load makes it difficult to detect in frail, elderly, and severely immune-compromised patients. In the present study, we developed a locked nucleic acid--based 1-tube nested real-time RT-PCR (OTNRT-PCR) assay with the advantages of extremely high sensitivity, facile operability, and less likelihood of cross-contamination. The sensitivity, specificity, and clinical performance of the OTNRT-PCR assay were compared in parallel with a conventional TaqMan probe-based real-time PCR (qRT-PCR) assay and a traditional 2-step nested RT-PCR assay. The limit of detection of the OTNRT-PCR assay was  $1.02 \times 10^{-1}$  TCID<sub>50</sub>/mL, equivalent to the traditional 2-step nested RT-PCR assay and 25-fold lower than the qRT-PCR assay. Of 616 nasopharyngeal aspirates tested, 143 RSV-negative samples by qRT-PCR were confirmed as positive by sequencing the OTNRT-PCR products. We therefore conclude that OTNRT-PCR is more sensitive than qRT-PCR for detection of RSV in clinical samples.

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## 1. Introduction

Human respiratory syncytial virus (RSV) is a frequent cause of respiratory infections worldwide, especially severe bronchiolitis and pneumonia in infants and young children, resulting in a comparatively long period of disease (Garg et al., 2016; Sato et al., 2005; Simoes, 1999). Frail, elderly, and severely immune-compromised patients and adults with cardiopulmonary disease are also at high risk of infection by RSV, with relatively low viral load, making it difficult to detect, and RSV constitutes a major cause of morbidity and mortality in these populations (Falsey et al., 2005; van Elden et al., 2003; Yunus et al., 2010). Therefore, a highly sensitive, rapid, accurate, and cost-effective assay for detection in patients infected by RSV is extremely important.

Molecular-based diagnostic methods for detecting RSV are commonly used, including traditional real-time PCR (qRT-PCR) (Do et al., 2012; Hu et al., 2003) and multiplex real-time PCR (Bonroy et al., 2007; Brittain-

Long et al., 2008; Sanghavi et al., 2012), which are sensitive and specific for detection of RSV. However, the detection limit of these assays for RSV is not yet adequate for assessing clinical specimens with a low viral load of RSV infection, resulting in false-negative diagnosis. The conventional 2-step nested RT-PCR assay (Bellau-Pujol et al., 2005; Coiras et al., 2003; Lam et al., 2007) has sufficient sensitivity but is nevertheless labor-intensive, time-consuming, and susceptible to cross-contamination, making it unsuitable for wide application in clinical settings.

In the present study, we developed a locked nucleic acid (LNA)-based 1-tube nested real-time RT-PCR (OTNRT-PCR) assay using melting curve analysis with the advantages of extremely high sensitivity, easy performance, and less likelihood of cross-contamination. The clinical performance of the OTNRT-PCR assay was also compared in parallel with a conventional TaqMan probe-based real-time PCR (qRT-PCR) assay and a traditional 2-step nested RT-PCR assay using clinical samples.

## 2. Materials and methods

## 2.1. Virus stock and clinical samples

RSV A (strain CC12-10) with an infectivity titer of  $2.00 \times 10^5$  50% tissue culture infective doses (TCID<sub>50</sub>)/mL was kindly provided by the National Laboratory for Poliomyelitis, Disease Control and Prevention,

\* Corresponding author. Tel./fax: +86-0311-85911189.

\*\* Corresponding author. Tel./fax: +86-010-58900810.

E-mail addresses: zhaoli0226@163.com (L. Zhao), wangji19871229@sina.com (J. Wang), 13832179762@139.com (G. Li), qiufangzhou91@sina.com (F. Qiu), conan0829@126.com (C. Chen), 1173791762@qq.com (M. Zhao), luka\_wl@163.com (L. Wang), dsx53228@163.com (S. Duan), 15131129999@139.com (Z. Feng), maxj@ivdc.chinacdc.cn (X. Ma).

<sup>1</sup> Li Zhao, Ji Wang, and Gui-xia Li contributed equally to the article.

Chinese Center for Disease Control and Prevention, and was used as a reference virus to evaluate the sensitivity of the OTNRT-PCR assay.

In total, 616 nasopharyngeal aspirates (NPAs) were collected from hospitalized patients with respiratory infection admitted to the Children's Hospital of Hebei, China, during September–November, 2017. The definition of respiratory infection is as follows: the patients presented with fever (temperature equal to or greater than 38 °C) and runny nose, in addition to 1 or more of the following symptoms: cough, myalgia, nasal congestion, headache, sore throat, and earache, within 5 days of the symptom onset. Of those, 251 (40.75%) were female and 365 (59.25%) were male, and ages ranged from 30 days to 11 years. A total volume of 0.5 mL NPA was collected in 3.5-mL transport medium and stored at –80 °C.

All aspects of the study were performed in accordance with national ethics regulations and approved by the Institutional Review Boards of National Institute for Viral Disease Control and Prevention, Center for Disease Control and Prevention of China. Children's parents were apprised of the study's purpose and of their right to keep information confidential. Written informed consent was obtained from parents or caregivers.

## 2.2. Nucleic acid extraction

Reference RSV stock and clinical samples were extracted using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Extracts were eluted in 50 µL of nuclease-free water and stored at –80 °C until needed.

## 2.3. Design of outer and inner primers for OTNRT-PCR

OTNRT-PCR primers designed for RSV were derived from previously published 2-step seminested primers (Bellau-Pujol et al., 2005) with base modifications (Fig. 1 shows the schematic description of the primer design). The sequences of both the inner and outer primers used in the OTNRT-PCR assay were the same as those of the 2-step seminested assay, while only the outer primers of the OTNRT-PCR assay were modified by LNA. A '+' symbol is added in front of modified bases in Table 1. The advantages of LNA modification include increased maximum primer annealing temperature without affecting specific matching between complementary strands and increased probe signal with limiting primer or Taq DNA polymerase. Primers modified by LNA also have higher analytical sensitivity and efficiency for DNA amplification (Ballantyne et al., 2008; Burbano et al., 2010; Chen et al., 2016; Latorra et al., 2003; Levin et al., 2006; Suresh and Priyakumar, 2013).

## 2.4. Determination of primer maximal annealing temperature

Gradient PCR was conducted with annealing temperatures ranging from 52 °C to 72 °C to determine the maximum range at which the outer primer, inner primer, and LNA-outer primer could operate. Gradient PCR was performed individually in volumes of 25 µL containing  $3.20 \times 10^2$  TCID50/mL RSV stock, outer, inner, or LNA-outer primers, and One Step RT-PCR Enzyme Mix (QIAGEN, Germany) on a Mastercycler

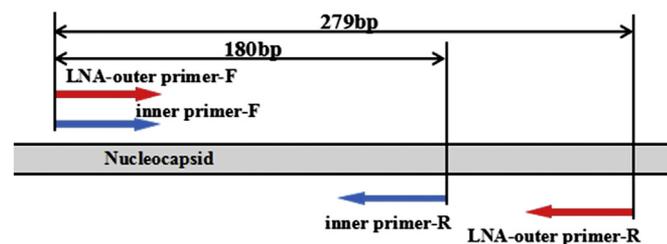


Fig. 1. Schematic description of primer design for the OTNRT-PCR assay. Detailed primer information is listed in Table 1.

Nexus Gradient PCR Amplifier (Eppendorf, Germany) under the following conditions: a 30-min reverse transcription step at 50 °C, a 15-min denaturation step at 95 °C, and 40 cycles at 94 °C for 1 min for annealing at various temperatures for 1 min, extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.

## 2.5. OTNRT-PCR and melting curve analysis

The OTNRT-PCR assay was performed in a 25-µL reaction system containing 5 µL of 5× PCR buffer, 2 µL of One Step RT-PCR Enzyme Mix (QIAGEN), 1 mM dNTP mix, 0.6 µM SYTO9 (Life Technologies, USA), 0.1 µL of RRI, 0.5 µL of LNA-outer primer mix (0.5 µM), 3 µL of inner primer mix (5 µM), and 2 µL of template nucleic acid using a CFX96 Real-Time PCR System (Bio-Rad, USA) under the following conditions: a 30-min reverse transcription step at 50 °C, a 15-min denaturation step at 95 °C, and 15 cycles at 94 °C for 30 s, 64 °C for 40 s, and 72 °C for 40 s, followed by 35 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s. Assays for dissociation were performed by incubating the reaction mixture at 65 °C for 5 s then increasing the temperature to 95 °C over a period of 20 min. Positive and negative controls were included in each run. PCR products were analyzed and confirmed by agarose gel electrophoresis (3.0% agarose gels; TSINGKE) to ensure that no undesirable DNA bands were observed, and that only the product of the predicted size was obtained.

## 2.6. Sensitivity of the OTNRT-PCR assay

Five-fold serial dilutions of viral RNA preparation from a reference RSV isolate ranging from  $8.00 \times 10^3$  TCID50/mL to  $2.05 \times 10^{-2}$  TCID50/mL were analyzed by the OTNRT-PCR assay as described above. Meanwhile, a traditional 2-step seminested RT-PCR assay and traditional TaqMan probe-based real-time PCR (qRT-PCR) were also performed in parallel using the same amount of template according to the published protocols (Bellau-Pujol et al., 2005; Sanghavi et al., 2012). The qRT-PCR results were defined as positive if the cycle threshold (Ct) value was not higher than 35.

## 2.7. Specificity of the OTNRT-PCR assay

The specificity of the OTNRT-PCR assay for RSV was evaluated using 315 out of 616 clinical specimens OTNRT-PCR detected RSV-negative in this study. These clinical samples were retrospectively tested using a Respiratory Pathogen 13 Detection Kit (13× kit, Health Gene Technologies, Ningbo, Zhejiang, China) (Zhao et al., 2017), which enables simultaneous detection of 13 respiratory pathogens including human rhinovirus (HRV), influenza virus types A (FluA), FluA-H1N1, FluA-H3, influenza virus types B (FluB), adenovirus, human Bocavirus, metapneumovirus (HMPV), parainfluenza virus (PIV), coronavirus (COV), respiratory syncytial virus (RSV), and mycoplasma pneumonia (MP) and Chlamydia (including CP and CT) in a single reaction.

## 2.8. Detection of clinical samples

The OTNRT-PCR assay for the detection of RSV was evaluated using a total of 616 NPAs selected from children hospitalized with respiratory infection. For comparison, traditional 2-step seminested RT-PCR and qRT-PCR assays were also performed in parallel. Sequencing of OTNRT-PCR products was performed to resolve discrepant results among the 3 assays. For detection of potential mixed infection, all the samples were retrospectively tested by 13× kit (Zhao et al., 2017).

## 2.9. Statistical analysis

IBM SPSS Statistics, version 21 (IBM Corporation, NY) was used to perform statistical analysis. The results of clinical detection by OTNRT-PCR assay, 2-step seminested RT-PCR, and qRT-PCR were analyzed

**Table 1**

Primers used for the OTNRT-PCR assay, the 2-step seminested RT-PCR assay, and the qRT-PCR assay.

Reference method	Sequence (5' to 3')	Primer length (bp)	Size (bp)	Product Tm (°C)	Gene	References
OTNRT-PCR	F <sup>a</sup> :GGAACAAGT+TGTT+GA+GGTTTATGAATATGC	30	279	78.5±0.5	N	This study (Bellau-Pujol et al., 2005)
	R <sup>a</sup> :TTCTGCTGT+CAA+GTCT+AGTACACTGTAGT	29				
	F:GGAACAAGTTGTTGAGGTTTATGAATATGC	30				
	R:GGTGTACCTCTGTA CTCTC	19				
Two-step seminested RT-PCR	F:GGAACAAGTTGTTGAGGTTTATGAATATGC	30	279	180	N	(Bellau-Pujol et al., 2005)
	R:TTCTGCTGTCAAGTCTAGTACACTGTAGT	29				
	F:GGAACAAGTTGTTGAGGTTTATGAATATGC	30				
	R:GGTGTACCTCTGTA CTCTC	19				
qRT-PCR	F:CACWGAAGATGCWAATCATAAATTCA	26	25	N	(Sanghavi et al., 2012)	
	R:GTATYTTTATRGTGCTTCYCTCTCAACC	30				
	P:FAM-TAATAGGTATGTTATATGCKATGTC-BHQ	25				

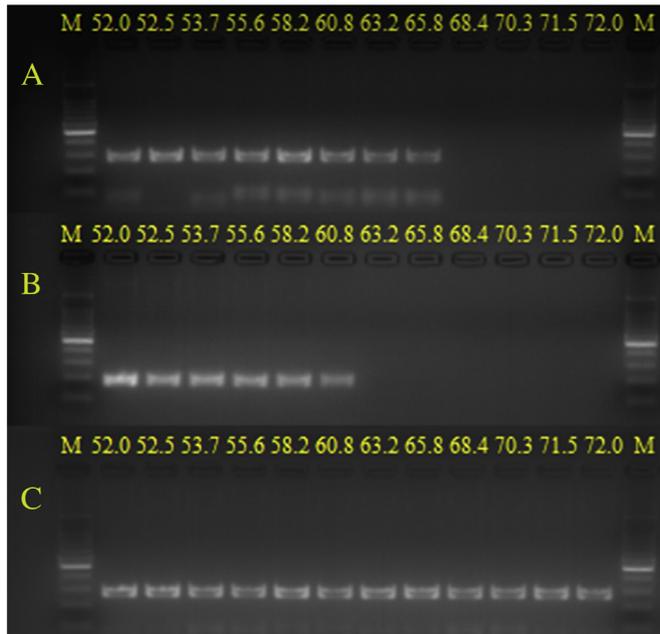
<sup>a</sup> A '+' symbol in front of nucleotides indicates an LNA monomer substitution.

using Kappa and McNemar's tests, and a value of  $P < 0.05$  was considered statistically significant.

### 3. Results

#### 3.1. Analysis of maximal primer annealing temperature

The results showed that the highest annealing temperatures of outer primers, inner primers, and LNA-outer primers were 65.8 °C, 60.8 °C, and 72.0 °C, respectively, using RNA from a  $3.20 \times 10^2$  TCID50/mL RSV stock (Fig. 2). The annealing temperature of the LNA-outer primer was dramatically increased compared with that of outer primer even though both primers had the same sequence. The optimal temperature selected for OTNRT-PCR was 52 °C for the inner primer and 64 °C for LNA-outer primer, with a difference of more than 10 °C between them, in an attempt to prevent competition between inner and outer primers during different PCR cycles in the OTNRT-PCR assay.



**Fig. 2.** Range of annealing temperature for outer primers, inner primers, and LNA-outer primers from 52.0 °C to 72.0 °C in gradient PCR using RNA from a  $3.20 \times 10^2$  TCID50/mL RSV stock as template. A, The highest annealing temperature of outer primers was 65.8 °C, yielding a 279-bp PCR product. B, The highest annealing temperature of inner primers was 60.8 °C, yielding a 180-bp PCR product. C, The highest annealing temperature of LNA-outer primers was 72.0 °C, yielding a 279-bp PCR product. M = 100-bp DNA markers.

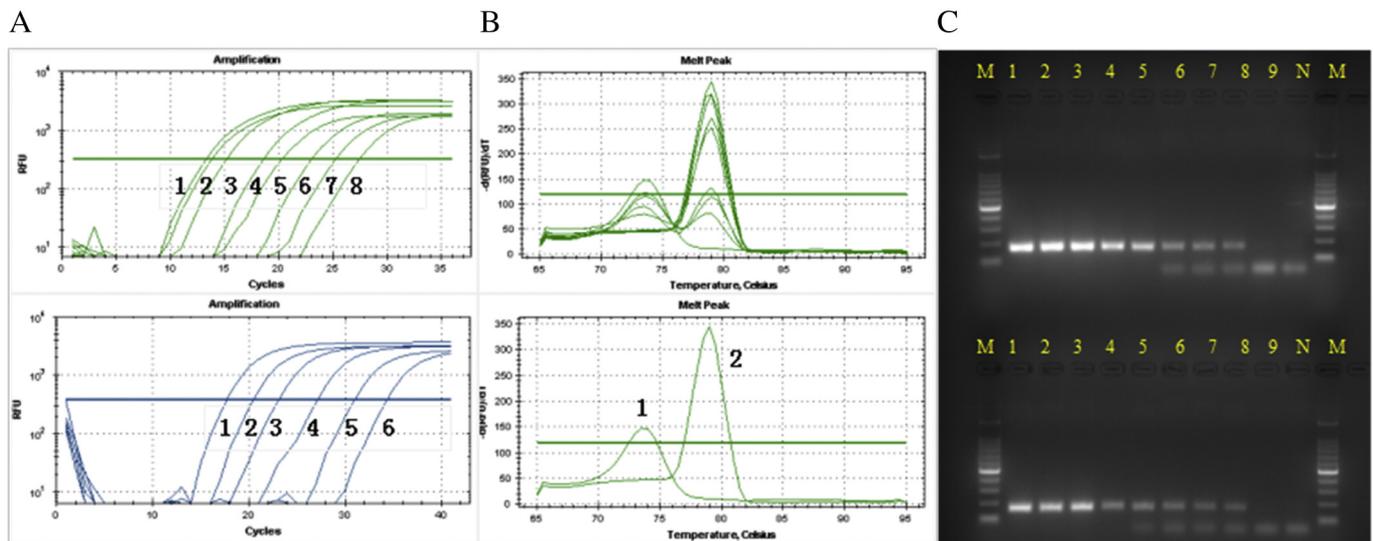
#### 3.2. Sensitivity and specificity of the OTNRT-PCR assay

Five-fold serial dilutions of RNA from the reference RSV (equal to  $8.00 \times 10^3$  TCID50/mL to  $2.05 \times 10^{-2}$  TCID50/mL) were tested in triplicate to ascertain the endpoint dilution at which the positive amplification signal (peak in the dissociation plot) was obtained in the OTNRT-PCR assay. As shown in Fig. 3, the limit of detection for the OTNRT-PCR assay was  $1.02 \times 10^{-1}$  TCID50/mL, equal to that of the traditional 2-step seminested RT-PCR assay and approximately 25-fold lower than that of the qRT-PCR assay ( $2.56 \times 10^0$  TCID50/mL). Analysis of the melting curves of amplification products showed that positive samples (180-bp product from inner PCR) generated an obvious peak in the dissociation plot at  $78.5 \pm 0.5$  °C, while negative controls produced a peak at 73.5 °C (primer dimers). Additionally, an extra melting peak (primer dimers) at 73.5 °C was observed when using template at a lower concentration. Both the OTNRT-PCR assay and traditional 2-step seminested RT-PCR assay products were further visualized by gel electrophoresis on a 3% agarose gel, and a single band (180 bp) was observed in the presence of a higher concentration of template, but primer dimer (<100 bp) was also generated in the presence of a lower concentration of template. The negative control (nuclease-free water) only yielded the primer dimer.

A total of 616 clinical samples were tested by OTNRT-PCR, of which 315 were negative for RSV. The negative specimens were used to determine the specificity of OTNRT-PCR in this study. These samples were retrospectively tested (Zhao et al., 2017) and confirmed to be positive for a variety of other respiratory pathogens including HRV, PIV, mycoplasma pneumonia (MP), human Bocavirus, adenovirus, COV, Chlamydia, FluA, influenza virus FluA-H3, HMPV, FluA-H1N1, and FluB. No unspecific amplification or detection by OTNRT-PCR was observed for these specimens, indicating high specificity for the OTNRT-PCR assay.

#### 3.3. Clinical evaluation using the OTNRT-PCR assay

The OTNRT-PCR assay, 2-step seminested RT-PCR assay, and qRT-PCR assay were individually tested to demonstrate the clinical performance for RSV RNA detection. A total of 616 clinical samples were tested in parallel, of which 315 were negative by the OTNRT-PCR assay and 2-step seminested RT-PCR assay. As shown in Table 2, both the OTNRT-PCR assay and the 2-step seminested RT-PCR assay detected 301 (48.86%, 301/616) of RSV samples, while only 158 (25.65%, 158/616) samples were RSV positive, with Ct values ranging from 18.01 to 34.48 by the qRT-PCR assay. The distribution of numbers of specimens with different Ct values analyzed by qRT-PCR for a total of 616 clinical samples was displayed in Fig. 4. The results were further analyzed statistically using Kappa and McNemar's tests. The results revealed discrepancies between the OTNRT-PCR assay and the qRT-PCR assay according to the Kappa coefficient, with Kappa values of 0.531 ( $P < 0.001$ ). Furthermore, in McNemar's tests, the 2 methods showed significant differences ( $\chi^2 = 141.007$ ,  $P < 0.001$ ). The 143 samples that were positive by OTNRT-PCR but negative by qRT-PCR were



**Fig. 3.** Comparison of the sensitivity of OTNRT-PCR, qRT-PCR, and 2-step seminested RT-PCR assays. A (top), Amplification curves of OTNRT-PCR assays using 5-fold serial dilutions of RSV isolates. A (bottom), Amplification curves of qRT-PCR assays using 5-fold serial dilutions of RSV isolates. B (top), Melting curve of SYTO9-based OTNRT-PCR assays using 5-fold serial dilutions of RSV isolates. B (bottom), Melting curves of the negative control with a melting peak at 73.5 °C (1), and the positive control (RSV isolate) with a melting peak at 78.5 ± 0.5 °C (2). C (top), OTNRT-PCR assay products tested by gel electrophoresis. C (bottom), Two-step seminested RT-PCR assay products tested by gel electrophoresis. Samples 1–9 represent the 5-fold serial dilution range ( $8.00 \times 10^3$ ,  $1.60 \times 10^3$ ,  $3.20 \times 10^2$ ,  $6.40 \times 10^1$ ,  $1.28 \times 10^1$ ,  $2.56 \times 10^0$ ,  $5.12 \times 10^{-1}$ ,  $1.02 \times 10^{-1}$ , and  $2.05 \times 10^{-2}$  TCID<sub>50</sub>/mL, respectively). N = negative control; M = 100-bp DNA markers.

confirmed as true positives by sequencing of OTNRT-PCR products. Besides, mixed infections involved in RSV and any other pathogens were found in 62 out of 158 (39.24%) qRT-PCR–positive samples and in 123 out of 301 (40.86%) OTNRT-PCR–positive samples, respectively. HRV was most frequently found in the mixed infection with RSV (data not shown).

#### 4. Discussion

LNA is a chemical modification which introduces a -O-CH<sub>2</sub>- linkage in the furanose sugar of nucleic acids and locks the conformation in a particular state (Rodriguez et al., 1994; Suresh and Priyakumar, 2013). LNA can be incorporated into DNA or RNA oligonucleotides to induce a local conformational change in the helix (Kaur et al., 2006). Upon LNA modification, the stability and affinity for DNA molecules are increased (Burbano et al., 2010), and the oligonucleotide melting temperature is also reportedly increased by LNA (Latorra et al., 2003). LNA modification has been used in many applications including single-nucleotide polymorphism analysis (Karmakar and Hrdlicka, 2013), real-time PCR probes (Osterback et al., 2013; Sun et al., 2007), antisense oligonucleotides (Wahlestedt et al., 2000), microarray probes (Castoldi et al., 2008), and PCR primers (Chen et al., 2016; Latorra et al., 2003). However, LNA modification has not been used in OTNRT-PCR by melting curve analysis. In the current study, we adapted and modified a previously described 2-step seminested RT-PCR process (Bellau-Pujol et al., 2005) and developed an LNA-based OTNRT-PCR assay.

In the OTNRT-PCR assay, the outer primers were modified by LNA to substantially increase the annealing temperature, resulting in a significant difference in the annealing temperature (64 °C for LNA-outer primers vs. 52 °C for inner primers), potentially allowing independent reaction during the amplification. In the initial cycles of the OTNRT-

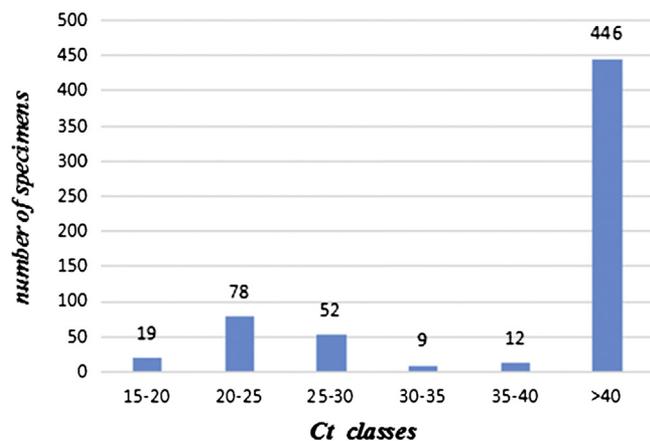
PCR assay, LNA-outer primers were annealed at a higher annealing temperature (64 °C), enabling hybridization of only the outer primers, and later cycles were carried out at a lower annealing temperature (52 °C), enabling hybridization of both the inner primers to the amplicons and the antisense oligonucleotides to the outer primers. The working concentration of each primer, the reaction parameters, and the running conditions of the OTNRT-PCR assay were optimized, and this enabled the detection of RSV with extremely high sensitivity while maintaining good specificity. Limiting the concentration of outer primers in PCR has been described as a way to improve the efficiency of 1-tube nested PCR (Erlich et al., 1991). In our study, LNA-outer primers were employed at a lower concentration than inner primers to minimize primer competition. The sensitivity of the commercial qRT-PCR kit in our study was  $2.56 \times 10^0$  TCID<sub>50</sub>/mL with a Ct value of 34.48 (Fig. 3A), which is commensurate with the reported values (Sanghavi et al., 2012). Meanwhile, the sensitivity of the developed OTNRT-PCR assay was  $1.02 \times 10^{-1}$  TCID<sub>50</sub>/mL, which is equal to that of the 2-step nested RT-PCR assay and 25-fold more sensitive than qRT-PCR. The high specificity of the OTNRT-PCR assay for RSV was also retrospectively evaluated using 315 RSV-negative clinical specimens that were positive for other respiratory pathogens.

This OTNRT-PCR assay was further evaluated and compared with 2-step seminested RT-PCR and qRT-PCR assays using 616 clinical samples. As shown in Table 2 and Fig. 4, 143 clinical samples detect by OTNRT-PCR assay were missed by qRT-PCR with CT > 35. Among them, 12 samples had a CT range of 35–40; 131 out of 446 samples were deemed to be negative for RSV by qRT-PCR but positive by OTNRT-PCR assay. This result suggests that the OTNRT-PCR assay is more sensitive than the qRT-PCR assay and retains the sensitivity of the 2-step seminested RT-PCR assay. It is possible that the higher sensitivity of the OTNRT-PCR assay could aid in the detection of clinical samples from adult

**Table 2**

The clinical performance of OTNRT-PCR and qRT-PCR compared with 2-step seminested RT-PCR as the reference method.

Assay	No. of clinical samples detected				Sensitivity (%)	Specificity (%)
	+/+	+/-	-/+	-/-		
OTNRT-PCR vs. 2-step seminested RT-PCR	301	0	0	315	100	100
qRT-PCR vs. 2-step seminested RT-PCR	158	0	143	315	52.49	100



**Fig. 4.** The distribution of numbers of specimens with different Ct values analyzed by qRT-PCR for a total of 616 clinical samples.

patients infected with RSV suffering severe disease and/or a lack of immunity, resulting in possible low virus load of RSV (Walsh et al., 2001). Thus, the OTNRT-PCR assay may not only benefit earlier diagnosis of RSV infection in children but also prove effective for detection in cases of low virus load, thereby improving disease symptoms in older patients.

PCR products could be readily detected by melting curve analysis using SYTO9 (Gudnason et al., 2007; Monis et al., 2005). Some studies have reported performing nested PCR in a single tube using fluorescent probes, gel electrophoresis, or spatial separation of outer and inner primer sets (Brisco et al., 2011; Dey et al., 2012; Hu and Arsov, 2014; Moser et al., 2012). However, our OTNRT-PCR assay can effectively prevent contamination and eliminate the need for post-PCR electrophoresis or the use of a fluorescent probe, and complete detection takes only 3 h. Furthermore, the running costs of the OTNRT-PCR assay are 10% less than the TaqMan probe qRT-PCR assay. The cost of the LNA-outer primers (~\$0.06/reaction) is similar to that of common primers, and the SYTO9 reagent is much cheaper than a fluorescent probe. Therefore, the proposed assay offers the advantages of being highly sensitive, real time, rapid, cost-effective, and contamination-free.

In this study, we only collected NPAs from hospitalized children as specimens; hence, a further evaluation of the performance of our assay for different types of specimen from different populations is needed in future work. In conclusion, the developed OTNRT-PCR assay for detection of RSV using primers modified by LNA was more sensitive than a corresponding qRT-PCR assay. Given the treat of RSV epidemics, the OTNRT-PCR assay could be used to detect RSV in patients earlier and could assist in the monitoring of therapy and disease prognosis, thereby reducing mortality in patients. Moreover, this LNA modification-based design provides a powerful strategy for converting existing 2-step nested PCR methods to OTNRT-PCR assays for other infectious agents.

#### Conflict of interest

All authors declare that they have no competing interests.

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#### Ethical approval

All aspects of the study were performed in accordance with national ethics regulations and approved by the Institutional Review Boards of the National Institute for Viral Disease Control and Prevention, Center for Disease Control and Prevention of China.

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