Analytical and Bioanalytical Chemistry (2023) 415:3131–3135
https://doi.org/10.1007/s00216-023-04704-9

COMMUNICATION

Reference material development for detection of human respiratory syncytial virus using digital PCR

Chunyan Niu[1] · Lianhua Dong[1] · Jiejie Zhang[1] · Di Wang[1] · Yunhua Gao[1]

Received: 27 February 2023 / Revised: 14 April 2023 / Accepted: 18 April 2023 / Published online: 9 May 2023
© Springer-Verlag GmbH Germany, part of Springer Nature 2023

Abstract

Nucleic acid testing is a powerful tool for the detection of various pathogens. Respiratory syncytial virus (RSV) is a major cause of acute respiratory infection, especially in young children and infants. To improve the confidence and reliability of nucleic acid testing results for RSV, reference materials (RMs) of both type A and B of RSV were developed by the National Institute of Metrology, China, code numbers NIM-RM 4057 and 4058. The reference material was composed of in vitro transcribed RNA containing the nucleocapsid (N) gene, matrix (M) gene, and partial polymerase (L) gene of RSV. A duplex reverse transcription digital PCR method was established with limit of blank (LoB), limit of detection (LoD) and limit of quantification (LoQ) of 2, 5, and 23 copies per reaction for RSV-A and 4, 8, and 20 copies per reaction for RSV-B. The certified value and expanded uncertainty (U, $k=2$) of the two RMs were determined to be $(6.1 \pm 1.4) \times 10^4$ copies/μL for RSV-A and $(5.3 \pm 1.2) \times 10^4$ copies/μL for RSV-B. The developed RMs can be used as standards to evaluate the performance of RSV detection assays.

Keywords Respiratory syncytial virus (RSV) · Digital PCR · Reference material · Nucleic acid testing

Introduction

Respiratory syncytial virus (RSV) is a common respiratory virus that can cause serious respiratory infections such as bronchiolitis and pneumonia, especially in young children [1]. RSV has also been recognized as a significant pathogen in adults with acute respiratory infection [2] and places a heavy burden on the healthcare system [3]. Increasing evidence has shown that outcomes for patients hospitalized with RSV are worse than for those with influenza or *Human metapneumovirus* (hMPV) [4]. More importantly, RSV infection can lead to higher rates of intensive care unit (ICU) admissions than influenza [4]. During the COVID-19 pandemic, RSV was the most common ($n=342$, 31.4%) virus identified in children co-infected with SARS-CoV-2 [5].

RSV is a non-segmented negative-strand RNA virus belonging to the family *Pneumoviridae*, genus *Orthopneumovirus* [6]. Its genome consists of approximately 15,200 nucleotides [7] and contains 10 genes that encode for 11 proteins [8]. RSV can be divided into two antigenic groups, A and B, and multiple genotypes within the two groups [9].

As molecular-based diagnostic methods are commonly used for detecting pathogens, nucleic acid testing has been known as the gold standard method for detecting acute RSV [2]. Various traditional real-time polymerase chain reaction (PCR) [10] and multiplex real-time PCR assays have been developed [11, 12]. Regardless of whether laboratory-developed assay or commercial assay is used, it is important to validate the assay and perform quality control throughout the detection process. Reference materials (RMs) are uniform and stable samples that can be used to ensure the accuracy and reliability of measurement results [13]. In this study, we developed a duplex reverse transcription digital PCR (RT-dPCR) method which can distinguish between RSV-A and RSV-B, and developed reference materials of RSV-A (NIM-RM 4057) and RSV-B (NIM-RM 4058) based on the RT-dPCR method.

✉ Chunyan Niu
niuchy@nim.ac.cn

[1] Center for Advanced Measurement Science, National Institute of Metrology, Beijing 100013, China

Materials and methods

Preparation of reference materials

The RM is composed of a single in vitro transcribed RNA construct (DNA was synthesized by Sangon, China) containing the complete nucleocapsid (N) gene, matrix (M) gene, and a segment of the polymerase (L) gene of RSV-A (NC_038235) and RSV-B (NC_001781), respectively (Fig. 1 and Table S1), in a matrix of 5 ng/μL total RNA extracted from cultured 293 T cells in buffered solution (RNA Storage Solution, Thermo Fisher Scientific, USA). In vitro transcription was performed using the MEGAscript™ T7 Transcription Kit (Thermo Fisher Scientific, USA). RNA transcripts were purified using the MEGAclear™ Kit (Thermo Fisher Scientific, USA). A total of 200 units of each RM were prepared, with each unit containing 50 μL. The approximate concentration for each of the RMs was 10^4 copies/μL.

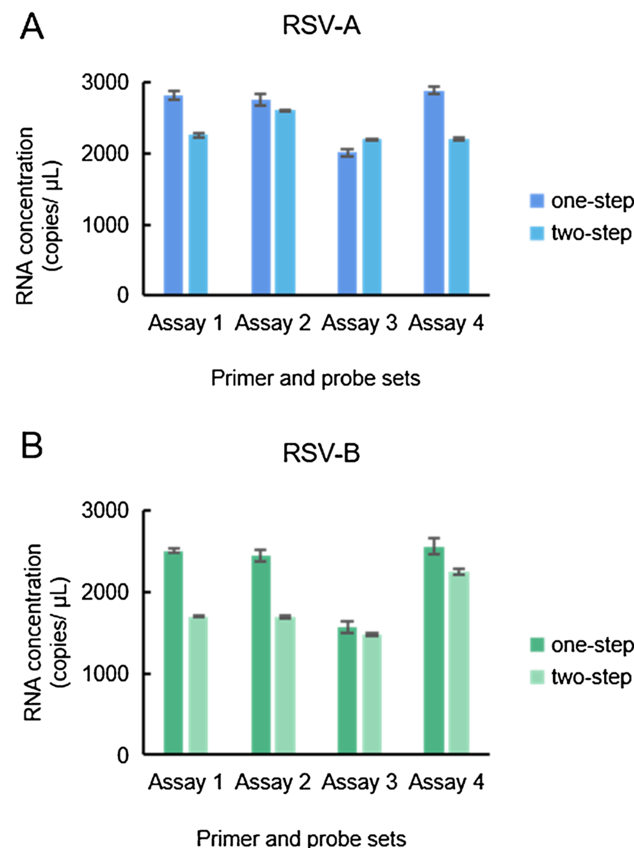

Fig. 1 Evaluation of four sets of assay using RNA template of RSV-A (**A**) and RSV-B (**B**) by one-step and two-step RT-dPCR

Performance of digital PCR method

The RT-dPCR was performed on a chip-based digital PCR system (DQ24, Sniper, China). One-step and two-step RT-dPCR methods were tested. For one-step RT-PCR, the reaction mixture included 11 μL of one-step SuperMix (Sniper), 2 μL of reverse transcriptase (Sniper), 1.1 μL mixture of primers and probe (final concentration of 500 nM and 250 nM), 6.4 μL of RNase-free double-distilled water (ddH_2O) and 1.5 μL of RNA template. The thermal cycling conditions were as follows: 50 °C for 20 min (reverse transcription); 95 °C for 10 min; 40 cycles of 95 °C for 30 s and 58 °C for 60 s; 60 °C for 1 min. For two-step RT-PCR, the reverse transcription step was performed first using the HiScript III Reverse Transcriptase kit (Vazyme, R302). The reaction mixture included 4 μL of 5× HiScript III Buffer, 1 μL of dNTP Mix (10 mM each), 1 μL of HiScript III Reverse Transcriptase (200 U/μl), 1 μL of RNase inhibitor (40 U/μl), 1 μL of gene-specific primers (10 uM each, Table 1), 2 μL of RNase-free ddH_2O and 10 μL of RNA template. The thermal cycling conditions were as follows: 50 °C for 15 min and 85 °C for 5 s. Complementary DNA (cDNA) was then used for dPCR. The reaction mixture included 11 μL of SuperMix (Sniper), 1.1 μL mixture of primers and probe (final concentrations of 500 nM and 250 nM), 8.4 μL of RNase-free ddH_2O, and 1.5 μL of cDNA. The thermal cycling conditions were as follows: 60 °C for 10 min; 95 °C for 10 min; 40 cycles of 95 °C for 30 s and 58 °C for 60 s; 60 °C for 1 min.

Assessment of homogeneity and stability

Ten units of each RM were assessed by RT-dPCR, and three replicates were analyzed per unit for homogeneity analysis according to ISO Guide 35 [14]. Short-term stability (STS) study was performed at a range of storage temperatures and times. The RMs were maintained at −70 °C (reference temperature), 4 °C, and 25 °C for 2, 5, and 7 days. Two replicate units were included per condition and assessed by RT-dPCR. Two replicates were analyzed per unit. The long-term stability (LTS) of the RMs was assessed by analyzing the RNA samples after storage for 1, 3, 5, and 13 months at the reference storage temperature of −70 °C. The sampling and analysis methods were the same as for STS. Trend analysis was used for the stability study according to ISO Guide 35.

Characterization of RMs and evaluation of measurement uncertainty

The RMs were characterized by the established RT-dPCR method. Five vials were quantified, with three replicates for

Table 1 Primer and probe sequences for RSV

Code	Target gene	Primer or probe	Sequence (5′-3′)	Genome location on RSV-A[a]	Genome location on RSV-B[b]	Reference
Assay 1	M	Forward primer	GGGCAAATATGGAAACATACGTG	3253–3275	3255–3277	[16]
		Reverse primer	TCTTTTTCTAGGACATTGTACTGAACAG	3310–3337	3312–3339	
		Probe	FAM-TTCACGAAGGCTCCACATA-TAMARA	3283–3301	3285–3303	
Assay 2	M	Forward primer	GGCAAATATGGAAACATACGTGAA	3254–3277	3256–3279	[17]
		Reverse primer	TCTTTTTCTAAGACATTGTAYTGAACAG	3310–3337	3312–3339	
		Probe	FAM-CTGTGTATGTGGAGCCTTCGTGAAGCT-BHQ1	3280–3306	3282–3308	
Assay 3	L	Forward primer	AATACAGCCAAATCCAACCAACTTTACA	13,850–13,877	13,861–13,888	[10]
		Reverse primer	GCCAAGGAAGCATGCAGTAAA	13,923–13,943	13,934–13,954	
		RSV-A-Probe	FAM-TGCTATTGTGCACTAAAG-MGB	13,899–13,916	-	
		RSV-B-Probe	VIC-CACTATTCCTTACTAAAGATGTC-MGB	-	13,905–13,927	
Assay 4	N	Forward primer	ATGGCTCTTAGCAAAGTCAAGT	1140–1161	1140–1161	[11]
		Reverse primer	TGCACATCATAATTRGGAGTRTCA	1238–1261	1238–1261	
		RSV-A-Probe	VIC-ACACTCAACAAAGATCAACTTCTRTCATCC AGCA-BHQ2	1170–1203	-	
		RSV-B-Probe	FAM-ACATTAAATAAGGATCAGCTGCTGTCATCC AGCA-TAMARA	-	1170–1203	

[a]Nucleotide numbering was based on type A RSV (GenBank accession number NC_038235)

[b]Nucleotide numbering was based on type B RSV (GenBank accession number NC_001781)

each vial. Data were analyzed by the Shapiro–Wilk test and Dixon and Grubbs tests. The arithmetic mean of the measured data was calculated as the certified value of the RM.

Uncertainties of RMs were calculated by taking into account the following factors: characterization of the RMs (u_{char}), homogeneity and LTS assessment of the RMs (u_{bb} and u_{lts}). Expanded uncertainties (U) were calculated by multiplying standard uncertainty (u) by a coverage factor (k) of 2 at 95% confidence level.

Results and discussion

Establishment of RT-dPCR for quantification and typing of RSV

According to previous reports, the most commonly tested target genes were N, M, and L. Four sets of primers and probes (Table 1) were tested and compared by one-step and two-step RT-dPCR using the in vitro transcribed RNA of RSV-A and RSV-B as templates (Fig. 1). The first two sets were universal for RSV and the last two sets were specific for type A and B RSV. As the three genes were constructed into the same molecule, theoretically their quantification results should be 1:1:1. Due to the difference in efficiency of amplification and reverse transcription, the latter being more critical especially for RNA, the four assay sets yielded different results. Both RSV-A and RSV-B obtained higher results by the one-step RT-dPCR than by the two-step RT-dPCR, except for assay 3 of RSV-A. For one-step RT-dPCR, the results of assay 4 were about 2.5% higher than assay 1, about 5% higher than assay 2, and about 40–60% higher than assay 3. Therefore, assay 4 was selected for the subsequent study for both type A and B RSV. The primer and probe concentration and annealing temperature were then further optimized, and the duplex RT-dPCR assay for simultaneous detection of RSV-A and RSV-B was established.

Validation of the RT-dPCR assay

The limit of blank (LoB), limit of detection (LoD), and limit of quantification (LoQ) for the RT-dPCR assays were determined to be 2, 5, 23 copies per reaction for RSV-A and 4, 8, and 20 copies per reaction for RSV-B according to EP 17A [15]. Sixty blank samples were used to determine the LoB (Table S2). Three or four levels of low-concentration samples with 20 samples for each level were used to determine the LoD (Table S3) and LoQ (Table S4).

The dynamic range of the assay was evaluated using serially diluted RNA samples. Ideal linearity was achieved over a 5-log dynamic range, from 10^1 to 10^5 copies per reaction, with R^2 of 0.9994 for RSV-A and 0.9991 for RSV-B (Fig. 2). The repeatability of the assay represented by the relative standard deviation (RSD) of copy number concentration ranged from 1.5% to 23.7% for RSV-A and 2.6% to 16.8% for RSV-B (Table S5). The specificity of the duplex assay was evaluated by cross-checking of RSV-A and RSV-B RNA, and no false-positive results were obtained (Fig S1).

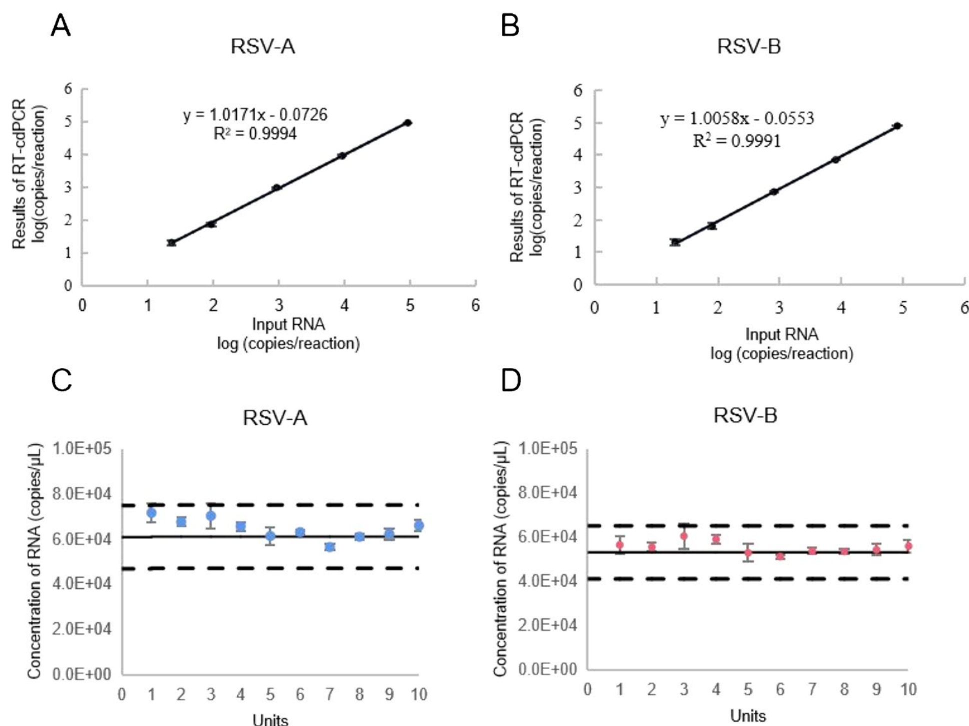

Fig. 2 Linearity of RT-dPCR assays for RSV-A (**A**) and RSV-B (**B**), and homogeneity of reference materials for RSV-A (**C**) and RSV-B (**D**). In (**C**) and (**D**), dots represent the means of each unit, solid lines indicate the reference value of the RMs, and dashed lines indicate the expanded uncertainty of the RMs

The established duplex RT-dPCR assay was confirmed to be sensitive for quantitative detection and subgroup-specific identification of RSV.

Homogeneity and stability of the RMs

Under the same conditions, ten units of each RM were assessed by RT-dPCR for homogeneity (Fig. 2, Table S6). The data did not indicate outlying unit means or any obvious trend in the filling sequence. The overall %CV of the data was 7.9% for RSV-A and 5.9% for RSV-B. We evaluated the between-unit standard deviation (s_{bb}) for each RM and found that s_{bb} was comparable with method repeatability standard deviation (s_r). Therefore, the RMs were considered sufficiently homogeneous, and s_{bb} was used as the estimate of standard uncertainty related to possible between-unit heterogeneity (u_{bb}).

STS study showed that there was no change in copy number concentration of RMs after storage at 4 °C and 25 °C for 7 days. LTS study showed that the two RMs were stable for as long as 13 months under −70 °C (Fig. S2 and Table S7). The LTS study data were plotted against storage time, and regression lines of RNA concentration (y_i) versus time (x_i) were calculated. The slope of the regression lines (β_1) and the standard deviation of the slope [$s(\beta_1)$] were then tested for statistical significance. The uncertainty contribution of LTS study (u_{lts}) was calculated as the product of the chosen shelf life and $s(\beta_1)$.

Characterization of the RMs

The certified value of the RM is represented as the copy number concentration of RNA determined by the established RT-dPCR method. The result of measurements are shown in Table 2. Shapiro–Wilk test showed that the data followed normal distribution. The Dixon and Grubbs tests showed no outliers. The certified values were calculated from the arithmetic mean of the five samples. The estimated relative standard uncertainty related to characterization ($u_{rel(\mathrm{char})}$) was calculated as

Table 2 Certified values and expanded uncertainties (U, $k=2$) of RMs

RMs	RT-dPCR results (copies/μL)	Relative standard deviation (RSD)	Certified value (copies/μL)	U ($k=2$) (copies/μL)
RSV-A	6.13E+04	4.23%	6.1	1.4
	6.30E+04			
	5.64E+04			
	6.10E+04			
	6.22E+04			
RSV-B	5.30E+04	2.22%	5.3	1.2
	5.12E+04			
	5.36E+04			
	5.35E+04			
	5.43E+04			

$$u_{rel(\text{char})} = \sqrt{u^2{}_{rel(\text{repeatibility})} + u^2{}_{rel(\text{volume})} + u^2{}_{rel(\text{dilution})}}$$

where $u_{rel(\text{repeatibility})}$ is the RSD of the measurement, $u_{rel(\text{volume})}$ is the uncertainty of the partition volume of dPCR (0.008) [18], and $u_{rel(\text{dilution})}$ is the calibration of the pipette (0.008).

The source of uncertainty for RMs mainly included characterization, homogeneity, and LTS study of the RM (Table S8). Finally, the certified value and expanded uncertainty (U, $k=2$) of the two RMs were determined to be $(6.1 \pm 1.4) \times 10^4$ copies/μL for RSV-A and $(5.3 \pm 1.2) \times 10^4$ copies/μL for RSV-B.

Conclusions

Several studies have shown that respiratory infection caused by RSV was associated with more severe risk and heavier hospital burden than expected. Nucleic acid testing plays a critical role in the detection of RSV, and it is important to develop certified reference materials to ensure accurate and reliable detection of RSV. A duplex RT-dPCR assay was established for absolute quantification and simultaneous detection of RSV-A and RSV-B. It was used in the development of new reference materials of RSV-A and RSV-B. The RMs were found to be homogeneous and stable, and can be used as standards to evaluate the performance of RSV detection assays.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-023-04704-9.

Funding This work was supported by the National Key Research and Development Program of China (2018YFC1200502).

Declarations

Conflict of interest The authors declare no competing interests.

References

1. CDC. https://www.cdc.gov/rsv/.
2. Busack B, Shorr AF. Going Viral-RSV as the Neglected Adult Respiratory Virus. Pathogens. 2022;11(11):1324.
3. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. N Engl J Med. 2005;352(17):1749–59.
4. Falsey AR, Walsh EE, House S, Vandenijck Y, Ren XH, Keim S, et al. Risk Factors and Medical Resource Utilization of Respiratory Syncytial Virus, Human Metapneumovirus, and Influenza-Related Hospitalizations in Adults-A Global Study During the 2017-2019 Epidemic Seasons (Hospitalized Acute Respiratory Tract Infection [HARTI] Study). Open Forum Infect Dis. 2021;8(11):ofab491.
5. Alhumaid S, Alabdulqader M, Al Dossary N, Al Alawi Z, Alnaim AA, Al Mutared KM, et al. Global Coinfections with Bacteria, Fungi, and Respiratory Viruses in Children with SARS-CoV-2: A Systematic Review and Meta-Analysis. Trop Med Infect Dis. 2022;7(11):380.
6. Nam HH, Ison MG. Respiratory syncytial virus infection in adults. BMJ. 2019;366:l5021.
7. Yu JM, Fu YH, Peng XL, Zheng YP, He JS. Genetic diversity and molecular evolution of human respiratory syncytial virus A and B. Sci Rep. 2021;11(1):12941.
8. Collins PL, Melero JA. Progress in understanding and controlling respiratory syncytial virus: still crazy after all these years. Virus Res. 2011;162(1–2):80–99.
9. Mufson MA, Orvell C, Rafnar B, Norrby E. Two distinct subtypes of human respiratory syncytial virus. J Gen Virol. 1985;66(Pt 10):2111–24.
10. Todd AK, Costa AM, Waller G, Daley AJ, Barr IG, Deng YM. Rapid detection of human respiratory syncytial virus A and B by duplex real-time RT-PCR. J Virol Methods. 2021;294:114171.
11. Wang L, Piedra PA, Avadhanula V, Durigon EL, Machablishvili A, Lopez MR, et al. Duplex real-time RT-PCR assay for detection and subgroup-specific identification of human respiratory syncytial virus. J Virol Methods. 2019;271:113676.
12. Feng ZS, Zhao L, Wang J, Qiu FZ, Zhao MC, Wang L, et al. A multiplex one-tube nested real time RT-PCR assay for simultaneous detection of respiratory syncytial virus, human rhinovirus and human metapneumovirus. Virology journal. 2018;15(1):167.
13. He HJ, Das B, Cleveland MH, Chen L, Camalier CE, Liu LC, et al. Development and interlaboratory evaluation of a NIST Reference Material RM 8366 for EGFR and MET gene copy number measurements. Clin Chem Lab Med. 2019;57(8):1142–52.
14. ISO Guide 35: 2017 Reference materials — Guidance for characterization and assessment of homogeneity and stability.
15. IFCC. EP 17 A-Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline.
16. Wenzhi Z, Qian Z, Jianmin W, Penghao X, Xiang W, Zhihui Z, et al. Development and application of tagman probe real-time PCR assay for detection of respiratory syncytial virus. Lett Biotechnol. 2012;23(2):211–4.
17. Fry AM, Chittaganpitch M, Baggett HC, Peret TC, Dare RK, Sawatwong P, et al. The burden of hospitalized lower respiratory tract infection due to respiratory syncytial virus in rural Thailand. PLoS ONE. 2010;5(11):e15098.
18. Dong LH, Meng Y, Sui ZW, et al. Comparison of four digital PCR platforms for accurate quantification of DNA copy number of a certified plasmid DNA reference material. Sci Rep. 2015;5:13174.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.