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RESEARCH ARTICLE

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Smaller reaction volume of triplex taqman real-time reverse transcription-PCR assays for diagnosing coronavirus disease 2019

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Abstract

Background: Coronavirus disease 2019 (COVID-19) has had a devastating impact on public health services worldwide. Currently, there are no standard remedies or therapies for COVID-19. it is important to identify and diagnose COVID-19 to control the spread. But clinical symptoms of COVID-19 are very similar to those of other respiratory viruses.

Results: As a result, the diagnosis of COVID-19 relies heavily on detecting pathogens. We established a bunch of triplex new TaqMan real-time PCR assays. Three sets of primers and probes (targeting the ORF1ab, N, and E genes, respectively) are poorly consistent with other human coronaviruses and the human influenza virus. The sensitivity of established PCR assays notices as few as 100 copies per PCR of the ORF1ab, N, and E genes. Meanwhile, standard curves concluded from constant PCR reaction all showed glorious linear correlations between Ct values and the polymer loading copy variety (correlation coefficient (R^2) of ORF1ab, N, and E genes is 0.996, 0.991, and 0.998, respectively). Surveillance of RNA-based pseudovirus demonstrated that they were identified to be positive with respect to SARS-CoV-2 and that established PCR assays are achievable.

Conclusion: The assays established provide a smaller reaction volume for diagnosing COVID-19.

KEYWORDS
COVID-19, nucleic acid testing, RT-PCR, SARS-CoV-2

1 | INTRODUCTION

The ongoing coronavirus disease (COVID-19), which is characterized by an extremely high infection rate and a relatively high mortality, is a pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It is challenging the medical community all over the world.[1] The SARS-CoV-2 is a positive-sense, single-stranded RNA betacoronavirus of size 29 kilobases (kb),[2] which shares ~80% and ~50% nucleotide identity with SARS-CoV and MERS-CoV, respectively,[3] and encodes a polyprotein (the open reading frame 1a and 1b, Orf1ab) and four structural

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proteins (envelope, E; membrane, M; nucleocapsid phosphoprotein, N; spike, S).[4] However, compared to SARS-CoV or MERS-CoV, SARS-CoV-2 spreads sooner making it troublesome to regulate.[5]

Several studies have shown that the majority of the COVID-19 patients can present with fever, cough, fatigue, headache, myalgia, and severe respiratory disease.[6–8] The most common manifestations of COVID-19 patients are almost similar to SARS and MERS.[9] Therefore, the diagnosis of COVID-19 is basically supported by the detection of pathogens. The World Health Organization and, therefore, the Chinese Centers for Disease Control have developed several RT-PCR protocols to diagnose COVID-19, which was helpful in identifying individuals at potential risk for SARS-CoV-2 transmission.[10] However, these applications are also critically affected by the high accuracy of the test. With respect to clinical decision-making, all of the causes of test failure led to erroneous diagnoses that gave rise to false positives or false negatives.

We established another set of triplex new TaqMan real-time PCR assays to identify SARS-CoV-2, and to conduct an evaluation of the availability of real-time PCR assays.

2 | MATERIALS AND METHODS

2.1 | Templates for experimental study

In this study, the reference sequences, available through the National Center for Biotechnology Information (NCBI; NC_045512.2, SARS-CoV-2), were synthesized (TsingKe Biotech, China) and provided templates for assay design. Target regions ORF1ab, N, and E DNA samples from SARS-CoV-2 have been amplified with the PCR specific primers (forward and reverse).

2.2 | The DNA-based plasmid preparation

Target regions of ORF1ab, N, and E, synthesized by TsingKe Biotech within the same vector (pcoldII), were subcloned within the plasmid (pET21a) using the EcoR I and Xho I restriction site. It was confirmed by next-generation sequencing (NGS), and subsequently reworked into the E. coli DH5α (Tiangen Biotech). The plasmid was exploited using TIANprep Mini Plasmid Kit (DP103, Tiangen Biotech), quantitated by ultraviolet (UV) light spectroscopy, and diluted by 10-fold gradient dilution for reserve use.

2.3 | RNA-based pseudovirus preparation

The target sequence of ORF1ab, N, and E was synthesized and cloned into a lentiviral vector and pseudovirus was prepared in 293T cells. This is a product called 2019-nCoV-Panel3 Pseudo virus Standard Reference (CBV30036) from Cobioer Biosciences (China). And its ORF1ab, N, and E copy number was 2.48×10^7 copies/ml, 1.84×10^7 copies/ml, and 5.85×10^7 copies/ml, respectively, quantitated by droplet digital PCR (DDPCR). At all stages, RNase contamination must be avoided by standard diethylpyrocarbonate (DEPC), employing disposable plastic, carrying gloves, etc. TaKaRa MiniBEST Viral RNA/DNA Extraction Kit Ver.5.0 (Code No. 9766, TaKaRa, Japan) was used to extract RNA from a 10-fold gradient dilution series of the pseudovirus. The RNA obtained above was reverse transcribed to the cDNA employing Goldenstar ™ RT6 cDNA Synthesis Kit (TSK301 M, TsingKe Biotech).

2.4 | Primers and probes

Three sets of primers and probes (targeting ORF1ab, N, and E gene, respectively), supported the ordination of SARS-CoV-2 (NCBI: NC_045512.2), were designed by NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Table 1 provides all primers and probes synthesized by TsingKe Biotech using the phosphoramidite method.

2.5 | TaqMan real-time RT-PCR assay

The novel PCR assay was developed by employing the 2xT5 Fast qPCR Mix (TSE301, TsingKe Biotech, China) on CFX384 Touch

TABLE 1 Primers and probes used for the TaqMan real-time RT-PCR assays

Gene	Primer/probe	Sequence (5′–3′)	5′ Modification	3′ Modification	Genomic location*	Amplicon (bp)
ORF1ab	Forward	CGCGAACCCATGCTTCAG	–	–	13424–13441	55
	Reverse	ACCGCAAACCCGTTTAAAAA	–	–	13479–13460	
	Probe	AGCTGATGCACAATC	FAM	BHQ1	13444–13458	
N	Forward	TGGACCCCAAAATCAGCGA	–	–	28285–28303	105
	Reverse	TTGTTTTGATCGCGCCCC	–	–	28390–28373	
	Probe	GCACCCCGCATTACGTTTGGTGGACCC	HEX	BHQ1	28307–28333	
E	Forward	CTTGCTTTCGTGGTATTCTTGC	–	–	26305–26326	88
	Reverse	CTCACGTTAACAATATTGCAGCA	–	–	26393–26371	
	Probe	AGCCATCCTTACTGCGCTTCGATTGTGTGC	ROX	BHQ1	26337–26366	

Note: *Numbering based on the SARS-CoV-2 reference genome (NC_045512.2).

TABLE 2 Efficiency of the TaqMan real-time RT-PCR assays

Gene	Mean Ct values at quantified plasmid copy number (copy/reaction)							Slope	R^2	Efficiency	Regression equation
	1×10^8	1×10^7	1×10^6	1×10^5	1×10^4	1×10^3	1×10^2				
ORF1ab	12.54 ± 0.118	16.14 ± 0.041	20.62 ± 0.254	24.35 ± 0.241	27.12 ± 0.060	30.54 ± 0.153	34.07 ± 0.211	−3.568	0.996	90.7%	Y=−3.568logX+41.465
N	13.44 ± 1.606	16.03 ± 0.082	19.21 ± 0.019	23.17 ± 0.018	25.25 ± 0.066	29.04 ± 0.129	30.71 ± 0.200	−3.091	0.991	110.6%	Y=−3.091logX+37.732
E	13.19 ± 0.030	16.03 ± 0.026	19.70 ± 0.055	23.11 ± 0.025	25.72 ± 0.315	29.64 ± 0.03	32.83 ± 0.195	−3.291	0.998	101.3%	Y=−3.291logX+39.344

Real-Time PCR Detection System (Bio-Rad). The optimum amount of primer, sensor quantity, and temperature were determined by a gradient test. Each 15 μl reaction mixture comprised 7.5 μl of 2 × T5 Fast qPCR Mix, 1.0 μl of 30.0 μM forward primer, 0.2 μl 30.0 μM reverse primer, 2.9 μl of 1.0 μM probe, 2.0 μl of total DNA, and 1.4 μl ddH2O. Thermal cycling included 95 °C for 2 min, then 45 cycles of 95°C for 10 sec, and 60°C for 50 sec. Fluorescence measurements were taken at 60°C throughout every cycle.

2.6 | Assessing sensitivity of assays

Continuous dilution of ORF1ab, N, and E plasmid (10^1–10^8 copies/ul) receptors in TE buffer solution was detected with established TaqMan real-time RT-PCR assay signature. In TaqMan real-time RT-PCR, ORF1ab, N, and E plasmids showed the highest dilution rates and all the replicates were positive.

2.7 | Evaluation of analytical specificity of assays

All primers and TaqMan probe sequences were confirmed by comparing the gene regions of all known SARS-CoV-2 sequences in the NCBI gene bank and several other human coronavirus and human influenza virus and flu virus reference sequences by MSFFT (v7.477) (SARS-CoV-2 (NC_045512.2, MW880890.1, MW880623.1), MERS (NC_019843.3, NC_038294.1), SARS (NC_002645.1), HCoV-229E (NC_005831.2), HCov_NL63 (NC_006577), HCoV-HKU1 (NC_004718), HCoV-OC43 (NC_006213), H1N1 (NC_002023), H7N9 (NC_026429)).

3 | RESULTS

3.1 | The developed TaqMan real-time RT-PCR assays are sensitive

In order to determine the sensitivity of the developed RT-PCR detection, the limit of detection was analyzed by 10-fold dilutions of plasmid from the E, N, and ORF1ab genes as models. Table 2 and Figure 1 demonstrate that the RT-PCR assay was sensitive, detecting as few as 100 copies per PCR of the ORF1ab, N, and E genes. The PCR assays were repeated thrice at 100 copies/reactions, each containing 94 measurements, and all the measurements were positive. The Ct values increase with copies of the ORF1ab, N, and E genes ranging from 1.0 × 108 to 1.0 × 10^2 copies in the reaction. The results show that the method produces trustworthy and repeatable amplification when the plasmid-based DNA model is 1.0 × 10^8 copies. Meanwhile, the standard curves of the same PCR reaction show that there is a good linear relationship between Ct values and the plasmid-based DNA loading copy number (correlation coefficient (R^2) of ORF1ab, N, and E genes is 0.996, 0.991, and 0.998, respectively). Table 2 presents the effectiveness of PCR

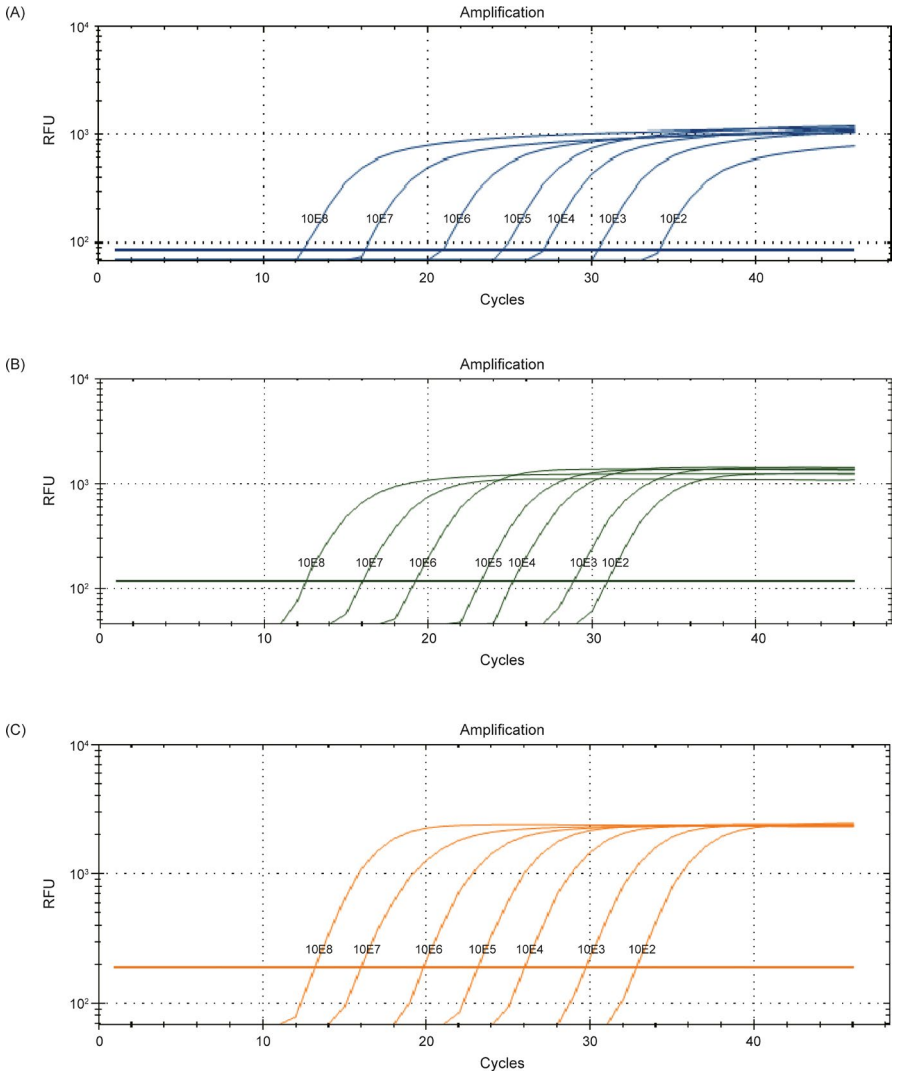

FIGURE 1 The PCR amplification curve originated from 10-fold dilution of plasmid from the ORF1ab gene assay (A), N gene assay (B), and E gene assay (C) of SARS-CoV-2

(E), the correlation coefficient (R^2), and the linear regression equation characterizing each standard curve. The regression equation was used to forecast the samples of unknown concentrations. The experimental results show that this method has high sensitivity and wide linear range.

3.2 | The developed TaqMan real-time RT-PCR assays are reproducible

In order to ascertain the reproducibility of PCR assays, intra-assay and interassay variabilities were assessed. The PCR assays were repeated thrice and linear regression analysis indicated that 100% repeatability was achieved when the range was greater than 100 copies/reactions for the ORF1ab, N, and E gene assays. The results of the correlational analysis are presented in Table 3. The coefficient of variation (CV) of Ct values within and between reactions was 0.224% ~1.231% and 1.134% ~6.503%, respectively, for the ORF1ab gene, 0.076% ~11.948% and 0.679% ~11.722%, respectively, for the N gene, and 0.011% ~1.223%, respectively, for the E gene. These suggest that the assays developed are highly reproducible.

3.3 | The developed TaqMan real-time RT-PCR assays are special

The specificity of TaqMan primers and probes was confirmed by comparing the gene regions of all known SARS-CoV-2 sequences in the NCBI gene bank. All signed primers and probe sequences are identical to all published sequences of SRAS-CoV-2. We lined up our primers and probes with the reference sequences of other human coronaviruses and flu viruses. Figure 2 shows that the aforementioned viral genome has poor consistency with primers and probes developed during this study, except for the SARS E gene. When the ORF1ab, N, and E genes were all positive in the assays, it was believed to be an infection with SARS-CoV-2. The latter suggests that the assays developed are special.

3.4 | The developed TaqMan real-time RT-PCR assays are confirmed by the RNA-based pseudovirus

RNA-based pseudovirus has been evaluated through PCR assays. Positive results were confirmed for SARS-CoV-2. It demonstrates that the PCR assays is achievable (Figure 3). When RNA was

TABLE 3 Reproducibility of the TaqMan real-time RT-PCR assays

Gene		Mean Ct values at quantified plasmid copy number (copy/reaction)							
		1×10^8	1×10^7	1×10^6	1×10^5	1×10^4	1×10^3	1×10^2	1×10^1
ORF1ab	CV within assay (%)	0.941	0.257	1.231	0.989	0.224	0.500	0.619	–
	CV between assay (%)	4.167	3.439	6.503	3.156	2.72	1.832	1.134	–
N	CV within assay (%)	0.650	0.444	0.260	0.076	0.098	0.514	11.948	–
	CV between assay (%)	11.722	1.183	1.439	2.552	1.175	0.679	2.116	–
E	CV within assay (%)	0.232	0.160	0.280	0.107	1.223	0.011	0.593	–
	CV between assay (%)	4.681	4.647	4.889	3.849	3.660	6.578	2.640	–

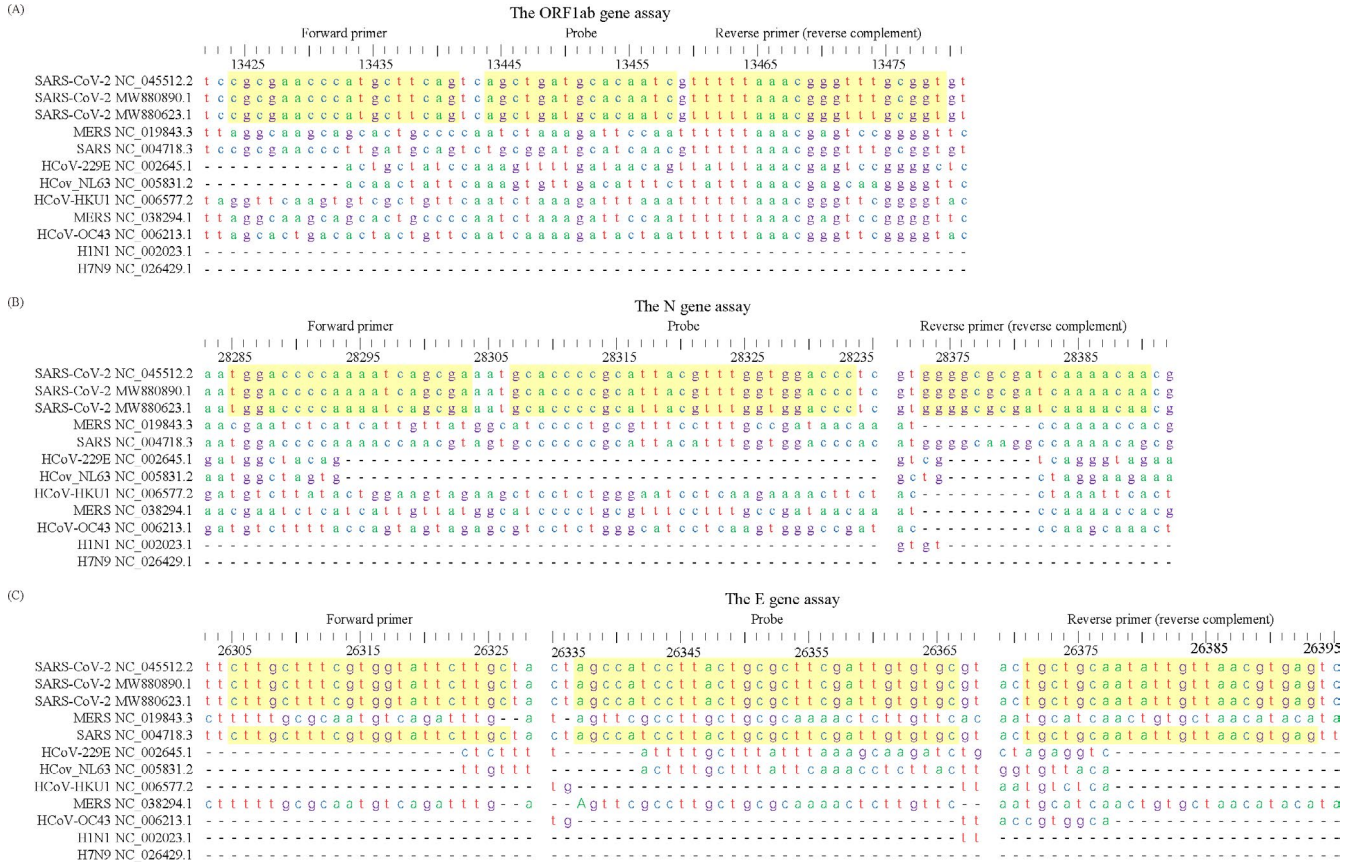

FIGURE 2 All primers and TaqMan probes sequences were confirmed by comparing the gene regions of known SARS-CoV-2 sequences, several other human coronaviruses, and flu viruses. The primers and probes of the ORF1ab gene assay (A), N gene assay (B), and E gene assay (C) of SARS-CoV-2 are aligned to the above sequence

extracted from a 10-fold gradient dilution series of the pseudovirus and was reverse-transcribed to the cDNA to determine the detection limit of the PCR assays, the results showed that the detection limit of ORF1ab, N, and E gene is 3.75×10^5, 2.76×10^5, 8.775×10^5copies/reactions, respectively. This is different from the detection limit of DNA plasmid as a template, perhaps because of the low performance of RNA extraction.

4 | DISCUSSION

Reported incidents of COVID-19 continue to escalate, posing a serious public health threat.[11] At present, RT-PCR, widely used throughout China and other countries, is still the standard for molecular diagnostics. And it is necessary for clinical identification and treatment and to prevent the unfolding of the sickness. A triplex real-time RT-PCR detection method for ORF1ab, N, and E genes in viral genome was developed. Sensitivity analysis resulted in a detection threshold of 100 copies per reaction. In addition, the strong correlation between RT-PCR and the gene regions of all known SARS-CoV-2 sequences in the NCBI gene bank provides us with confidence that these expressions are reproducible and specific. The resultant amplification efficiency ranged from 90.7% to 110.6%, showing that triplex real-time RT-PCR assays were well-optimized.

In order to improve the accuracy of COVID-19 detection, we developed a group of triplex new TaqMan real-time PCR assays. In the

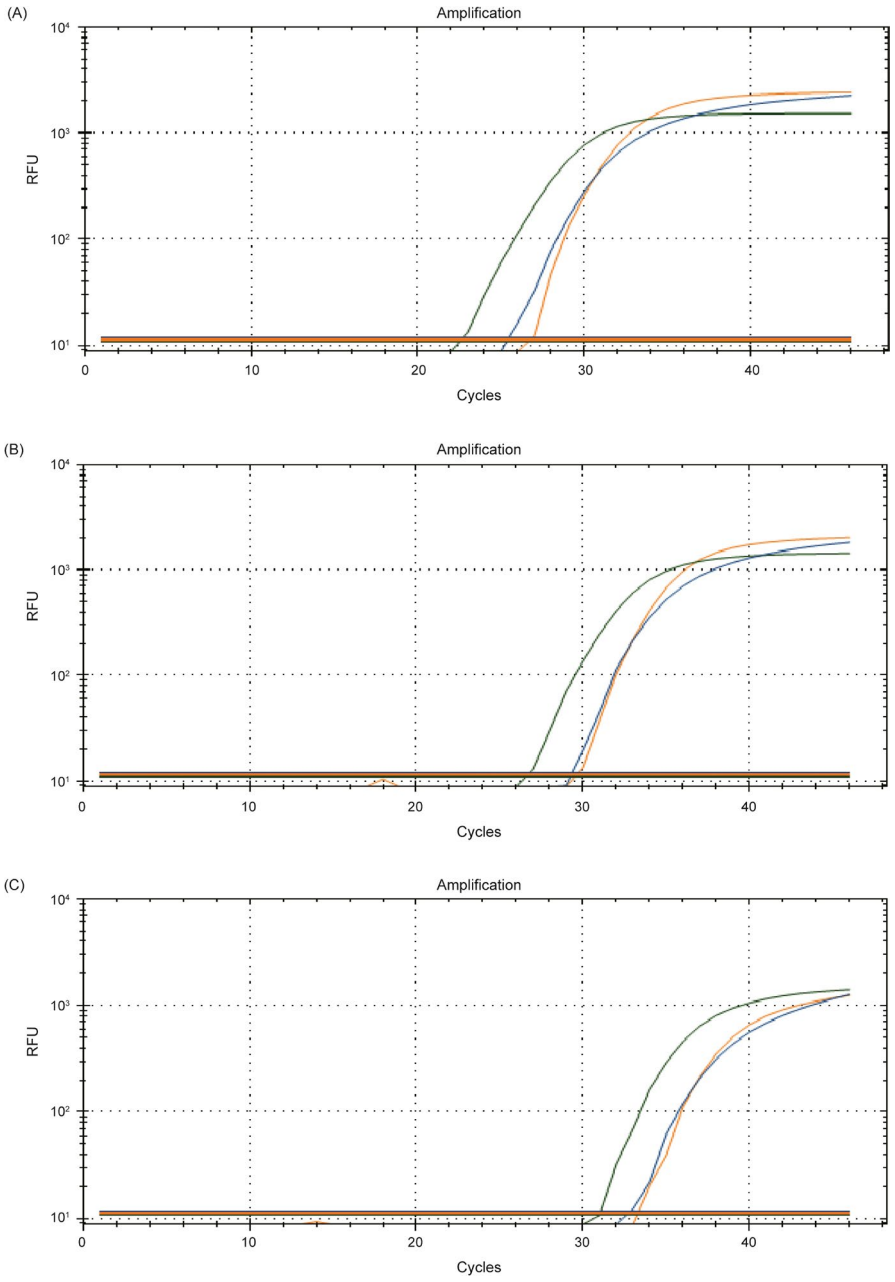

FIGURE 3 The PCR amplification curve originated from 10-fold gradient dilution series of the SARS-CoV-2 pseudovirus transcripts. (A) The PCR amplification curve of ORF1ab, N, and E genes of the SARS-CoV-2 pseudovirus transcripts which are 3.75×10^7, 2.76×10^7, 8.775×10^7 copies/reactions, respectively. (B)The PCR amplification curve of ORF1ab, N, and E genes of the SARS-CoV-2 pseudovirus transcripts which are 3.75×10^6, 2.76×10^6, 8.775×10^6 copies/reactions, respectively. (C) The PCR amplification curve of ORF1ab, N, and E genes of the SARS-CoV-2 pseudovirus transcripts which are 3.75×10^5, 2.76×10^5, 8.775×10^5 copies/reactions, respectively. The blue, green, and orange curves are the PCR amplification curves of ORF1ab, N, and E genes, respectively

specific analysis, the E gene primer and probe is aligned with SARS-CoV, because the homology between SARS-CoV-2 and SARS-CoV exceeds 80%[3] and the E gene is shorter,[12] which leads to the choices for the primer and probe of E Gene being limited. When the Ct value is less than 35, COVID-19 is diagnosed when all three channels are positive. A Ct value greater than 35 was interpreted as a weak positive. It may be due to a minor asymmetry between the primer/probe and the human genome model or other sources. When the Ct value is higher than 35, the results are confirmed by repeated tests, thus correcting the problem.

We developed a triplex TaqMan real-time PCR assay with a smaller volume of reaction mixture. This helps to identify and diagnose COVID-19 quickly, accurately, and on a preferred basis. Two TaqMan real-time RT-PCR assays, developed by Liu et al, have an N gene detection limit of 250 copies/ml.[13] The sensitivity of commercial kits ranges from 10^2 to 10^3 copies/ml. However, the kits are in excess of 20 ul/reaction. This indicates that the volume of the reactive mixture may affect the detection method boundary. The potential links between these effects should be further developed. When the TaqMan real-time RT-PCR tests developed are confirmed by the RNA-based pseudovirus, the detection limit is different than the detection limit for the DNA plasmid as a model. This can be caused by the poor performance of RNA extraction. The different methods of RNA extraction are also important factors which influence the detection limits of different commercial kits.

The TaqMan RT-PCR real-time assay, a major detection of COVID-19, is often used in clinical practice as nasopharyngeal swabbing is more manageable. However, the viral loads of different infected populations, as well as those of the same infected person at

different airway locations and disease progression points, are different.[14,15] And unreliable test reagents and inconsistent test operations are the major reasons for the misdiagnosis. The functioning of different persons may influence the results of tests, leading to the appearance of false positives or false negatives. For improving diagnostic accuracy of COVID-19, it is necessary to prevent the degradation of RNA in the sample, and to sample multiple parts of the infected person at the same time, supplemented by imaging or serological examination.

In conclusion, we have developed triplex real-time TaqMan RT-PCR tests that help to identify and diagnose COVID-19 quickly, accurately, and on a preferred basis.

AUTHOR CONTRIBUTIONS

The authors have contributed equally to this work. Longli Kang and Zhipeng Zhao were responsible for design. Zhiying Zhang, Jing Li, Junli Liu, Wenxue Dong, and Xu Yang were responsible for implementing and analyzing data. Wenxue Dong wrote the manuscript. Longli Kang provided insight on structuring the manuscript, and all authors contributed to manuscript completion.

DATA AVAILABILITY STATEMENT

The datasets supporting the conclusions of this article are included within the article.

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