Original Article

DOI: 10.5582/ddt.2022.01092

A fast RT-qPCR system significantly shortens the time for SARS-CoV-2 nucleic acid test

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SUMMARY Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a serious threat to global development. Rapid and accurate diagnosis is critical for containing the pandemic and treating patients in time. As the gold standard for SARS-CoV-2 diagnosis, the qualitative reverse transcription-PCR (RT-qPCR) test has long been criticized for its long detection time. In this study, we optimized the primers and probes targeting SARS-CoV-2 *ORF1ab* and *N* gene designed by the Chinese Center for Disease Control and Preventions (CDC) to increase their Tm values to meet the optimal elongation temperature of Taq DNA polymerase, thus greatly shortened the elongation time. The higher elongation temperature in turn narrowed the temperature range of the reaction and saved more time. In addition, by shortening the distance between the fluorophore at the 5' end and the quencher in the middle we got a probe with higher signal-to-noise ratio. Finally, by using all these measures and optimized RT-qPCR program we successfully reduced the time (nucleic acid extraction step is not included) for nucleic acid test from 74 min to 26 min.

Keywords SARS-CoV-2, detection time, Tm value, elongation temperature, probe

1. Introduction

The coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (*1*), is still spreading globally since the first case was reported in Wuhan, China, in December 2019 (*2*). As of December 18, 2022, an estimated 647.97 million people had been infected and more than 6.64 million have died (*3*). Although several kinds of vaccines and therapeutics have been developed and come into use (*4*), rapid and accurate diagnosis is always critical for containing the pandemic and treating patients in time (*5,6*). In China, due to the dynamic zero-out policy, large-scale screening of millions of people in an entire local region is occasionally needed to identify the latent infected persons in the population (*7*). Higher efficient detection means faster containment of the spread of SARS-CoV-2 and less economic loss.

Currently, nucleic acid test (NAS) and antigen tests are the two main methods for COVID-19 diagnosis and of which NAS is the main diagnostic method due to its higher sensitivity (*8,9*). Based on technical principle, NAS can be divided into two categories: one is targeted nucleic acid amplification testing (TNAAT), mainly including qualitative reverse transcription-PCR (RT-qPCR) and isothermal amplification (*10*); the other is direct nucleic acid testing without targeted amplification, such as nucleic acid hybridization, gene chip (*11*). Among all these nucleic acid test methods, RT-qPCR technology has the highest sensitivity, reliability and accuracy, thus become the gold standard for SARS-CoV-2 diagnosis (*12*).

RT-qPCR reaction includes two steps: reverse transcription and PCR cycle amplification (*13*). The routine program combines annealing and elongation into one step and adopts two-step cycle amplification to reduce the frequency of temperature rise and cooling, which requires a compromise between the annealing temperature of primers and the elongation temperature of Taq DNA polymerase. Generally, this process takes more than 70 min making a bottleneck of the diagnosis (*14*). In previous studies, rapid PCR was achieved

mainly through the improvement of DNA polymerase, reaction buffer and temperature control instruments. At present, the performance of Taq DNA polymerase used in RT-PCR detection has been quite excellent. And due to the popularity of RT-PCR technology, it would cost a lot to replace detection instruments in the majority of detection institutions. In this research, by optimizing the primer and probe design as well as the whole procedure we shortened the whole RT-qPCR time (nucleic acid extraction step is not included) 74 min to 26 min without sacrificing the sensitivity and accuracy. Our method provides a new way to shorten the PCR time.

2. Materials and Methods

2.1. Preparation of RT-qPCR template

225 μL SARS-CoV-2 *ORF1ab-N* pseudovirus (Sangon Biotech Co., Ltd, Shanghai, China) with the initial concentration of 10^8 copies/mL was added to a throat swab and placed into 3 mL preservative solution (25 mM Tris-HCl pH 7.6, 1 mM EDTA, 20 mM Guandine thiocyanate) to simulate clinically positive samples. Then 200 μL preservation solution was taken out for RNA extraction using MiniBEST Viral RNA Extraction Kit (Takara Biomedical Technology Co., Ltd, Beijing, China) and the target nucleic acid was eluted from the purification column with 30 μL enzyme-free water. Finally, 4 μL of RNA solution was used in the final reaction volume of 20 μL. According to the conversion, the pseudovirus concentration was 10^7 copies/mL in the RT-PCR system. The target nucleic acid was diluted with enzyme-free water, and the pseudovirus concentrations in the RT-PCR system were 10^6 copies/mL, 10^5 copies/mL, 10^4 copies/mL, 10^3 copies/mL, 700 copies/mL, 500 copies/mL, 300 copies/mL, and 100 copies/mL, respectively.

2.2. Determination of Tm value of primers and probes

The primers, probes to be tested and the single-stranded DNA fragments (template strand) to which they bind were synthesized by Sangon Biotech Co., Ltd. and purified by high performance liquid chromatography (HPLC). The volume of system for test was 20 μL, including 2.5 μM oligonucleotide, 2.5 μM template strand and 1× SYBR green1 dye (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The reaction was carried out on the SLAN-96P RT-qPCR instrument (Hongshi Medical Technology Co., Ltd., Shanghai, China) according to the following procedures: 95°C for 10 min, 30°C for 2 min, 65 cycles, each cycle increased by 1°C, and fluorescence was collected in each cycle. The standard melting curve of DNA fragments was measured and the reciprocal curve was obtained. Take the temperature corresponding to the peak of the reciprocal curve as the Tm value of the primers.

2.3. Comparison of quenching efficiency of probes

The probe to be tested and the corresponding probe labeled only with 5'-fluorophore were synthesized by Sangon Biotech Co., Ltd. and diluted to 10 μM. The fluorescence of the probe at 250 nM (the common concentration of probes in RT-qPCR reaction) was measured on a microplate spectrophotometer (Tecan Trading AG, Mannedorf, Switzerland), and the quenching efficiency of the probe was evaluated by comparing the fluorescence intensity with that of the corresponding probe labeled only with 5'-fluorophore.

2.4. RT-qPCR assay

The reaction system consisted of 10 μL 2× TaqMan Fast Master Mix, 1 μL Taq DNA polymerase (Vazyme Biotech Co.,Ltd, Nanjing, China), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.5 μL probe (10 μM), 3.5 μL nuclease-free water and 4 μL nucleic acid sample. RT-qPCR assay was performed on qTOWER3 (Analytik Jena AG, Jena, Germany). The procedure settings of four detection schemes were as follows. Scheme 1: reverse transcription at 55°C for 15 min, pre-denaturation at 95°C for 30 s, denaturation at 95°C for 10 s, annealing and elongation at 60°C for 30 s, 45 cycles. Scheme 2: reverse transcription at 55°C for 15 min, pre-denaturation at 95°C for 30 s, denaturation at 95°C for 10 s, annealing and elongation at 70°C for 30 s, 45 cycles. Scheme 3: reverse transcription at 55°C for 15 min, pre-denaturation at 95°C for 30 s, denaturation at 95°C for 10 s, annealing and elongation at 70°C for 2 s, 45 cycles. Scheme 4: reverse transcription at 55°C for 2 min, pre-denaturation at 95°C for 2 s, denaturation at 95°C for 1 s, annealing and elongation at 70°C for 2 s, 41 cycles.

2.5. Determination of the limit of detection

A scheme was performed with 5 template concentrations of 10^3 copies/mL, 700 copies/mL, 500 copies/mL, 300 copies/mL and 100 copies/mL, and each concentration was repeated 20 times. The template concentration with a positive rate greater than 95% (19 times) was defined as the limit of detection of the detection scheme. The criteria for judging the results were as follows. Positive results: cycle threshold (CT) values of FAM and VIC channels were both less than or equal to 38. Negative results: CT values of FAM and VIC channels were both greater than 38. Suspicious results: The CT value of one detection channel was less than or equal to 38, and the CT value of the other channel was greater than 38. Repeated testing was required for the suspicious results. And if the results were still consistent with the previous results, the samples were judged as negative.

2.6. Determination of the detection specificity

The long primers and long probe we designed were improved on the basis of the primers and probe targeting SARS-CoV-2 *ORF1ab* and *N* gene designed by China CDC, and the data of various detection institutions in China have proved the excellent specificity of these primers. In the evolution analysis of SARS-CoV-2, the viruses genetically similar to the SARS-CoV-2 genome sequence are the other six human coronaviruses. Therefore, we cloned the corresponding detection genes of various human coronaviruses into plasmids as RT-qPCR templates for detection specificity analysis. The data was obtained from triplet experiments.

2.7. Determination of the detection repeatability

To assess the repeatability of the three-channel fast RT-qPCR system, five replicates were executed within and between groups with three pseudovirus concentrations (10^6 copies/mL, 10^5 copies/mL and 10^4 copies/mL). The coefficient of variation (CV) was calculated according to the Ct value of the test results, and the calculation method was: CV = standard deviation/mean ×100%.

2.8. Statistics analysis

All experiments were repeated independently three times in order to minimize the effect of random initialisation, and we reported the mean and standard deviation (SD) of the experiments. The Student's *t*-test by SPSS 15 was used to perform data analysis. Statistical significance was assessed based on the p-value: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

3. Results

3.1. Primers and probe designed by China CDC cannot work at the optimal elongation temperature of Taq DNA polymerase

The Tm values of CDC-*ORF1ab* PF, CDC-*ORF1ab* PR, CDC-*ORF1ab* Probe, CDC-*N* PF, CDC-*N* PR and CDC-*N* Probe were 68.49 ± 0.04°C, 65.29 ± 0.06°C, 71.85 ± 0.02°C, 64.66 ± 0.05°C, 66.52 ± 0.09°C, and 71.81 ± 0.02°C, respectively (Figure 1). This implied that PCR system containing these primers and probes will not work well at the optimal elongation temperature (70-74°C) of Taq DNA polymerase. This speculation was then confirmed by the lack of amplification curve after 45 cycles of PCR with an elongation temperature of 70°C. This result suggested that in order to make Taq DNA polymerase work at the optimal elongation temperature of about 70°C the primers and probe must be modified.

3.2. Primers and probe modification

In order to get primers and probes that can be used at the optimum elongation temperature of Taq DNA polymerase, an array of derivatives of the China CDC primers targeting SARS-CoV-2 *ORF1ab* and *N* gene were designed and tested. The best pair of primers and probes were named as long-*ORF1ab* PF, long-*ORF1ab* PR, long-*ORF1ab* Probe, long-*N* PF, long-*N* PR and long-*N* Probe, respectively (Table 1). Their Tm values measured 72.49 ± 0.03°C, 73.91 ± 0.01°C, 78.82 ±

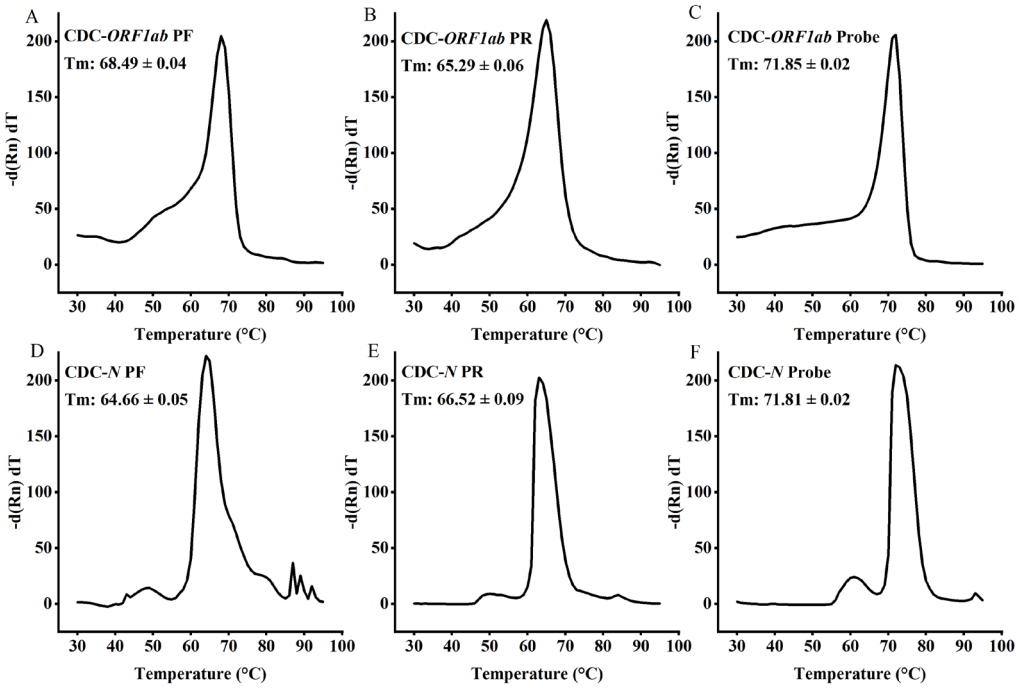

Figure 1. The melting curves of primes and probes designed by China CDC.

0.06°C, 73.22 ± 0.07°C, 73.77 ± 0.03°C and 77.63 ± 0.04°C, respectively (Figure 2). To monitor the sampling step of nucleic acid detection, we designed detection primers and probe targeting human ribonuclease P (*RP*) gene based on the same principle, named long-*RP* PF, long-*RP* PR and long-*RP* Probe. And their Tm values were 71.33 ± 0.02°C, 73.49 ± 0.08°C and 79.56 ± 0.05°C, respectively. Experiments indicated that these pairs of primers worked well at the elongation temperature of 70°C.

The commonly used Taqman probe has a 5'-fluorophore and 3'-quencher, named FQ probe. The probes (long-*ORF1ab* Probe, long-*N* Probe and long-*RP* Probe) with higher Tm value means longer distance between the fluorophore and the quencher. This would greatly reduce the quenching efficiency and thus the signal-to-noise ratio (*15*). In order to solve this problem, the design of the probe must be changed. Taking long-*ORF1ab* Probe as research object, we then designed two kings of probes for test: both probes have a fluorophore at 5' end and a quencher (BHQ1) at thymine 10 bases from the 5' end with the 3'-OH of probes sealed with another BHQ1 (for FQQ Probe) or Spacer C3 (for FQS Probe) to prevent elongation (Table 1). Experiment showed that both probes have a fluorescent background of 1% indicating a quenching efficiency of 99% for both (Figure 3). Under the same reaction conditions, the background signal of FQS Probe and FQQ Probe

Table 1. The sequences of primers and probes used in RT-qPCR test

Primers	Sequence
CDC-*ORF1ab* PF	5'-CCCTGTGGGTTTTACACTTAA-3'
CDC-*ORF1ab* PR	5'-ACGATTGTGCATCAGCTGA-3'
CDC-*ORF1ab* Probe	5'-VIC-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1-3'
CDC-*N* PF	5'-GGGGAACTTCTCCTGCTAGAAT-3'
CDC-*N* PR	5'-CAGACATTTTGCTCTCAAGCTG-3'
CDC-*N* Probe	5'-FAM-TTGCTGCTGCTTGACAGATT-BHQ1-3'
long-*ORF1ab* PF	5'- GACCCTGTGGGTTTTACACTTAAAAACACAGTCTGT-3'
long-*ORF1ab* PR	5'- AACGATTGTGCATCAGCTGACTGAAGCATGGGT-3'
long-*ORF1ab* Probe	5'-VIC- CCGTCTGCGGTATGTGGAAAGGTTATGGCTGTAGTTGTGATCAACTCCGC-BHQ1-3'
long-*ORF1ab* FQQ Probe	5'-VIC-CCGTCTGCGGT-BHQ1-ATGTGGAAAGGTTATGGCTGTAGTTGTGATCAACTCCGC-BHQ1-3'
long-*ORF1ab* FQS Probe	5'-VIC-CCGTCTGCGGT-BHQ1-ATGTGGAAAGGTTATGGCTGTAGTTGTGATCAACTCCGC-Spacer C3-3'
long-*N* PF	5'- CCAGGCAGCAGTAGGGGAACTTCTCCTGCTAGAATGGC-3'
long-*N* PR	5'- GGCCTTGTTGTTGTTGGCCTTTACCAGACATTTTGCTCTCAAGCTG-3'
long-*N* Probe	5'-FAM-TGGCGGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAG-BHQ1-3'
long-*N* FQS Probe	5'-FAM- TGGCGGTGAT-BHQ1-GCTGCTCTTGCTTTGCTGCTGCTTGACAG-Spacer C3-3'
long-*RP* PF	5'-CCTCGGCCATCAGAAGGAGATGAAGATTGTCTTCCAGCTTCCA-3'
long-*RP* PR	5'-GAGCCCAAGAGGCAAAGGTTGCAGTGAGCCGAGATTG-3'
long-*RP* FQS Probe	5'-ROX-TGGTCTCACT-BHQ2-CTGTCACCCAGGCTGGAGTGCAGTGGC-Spacer C3-3'

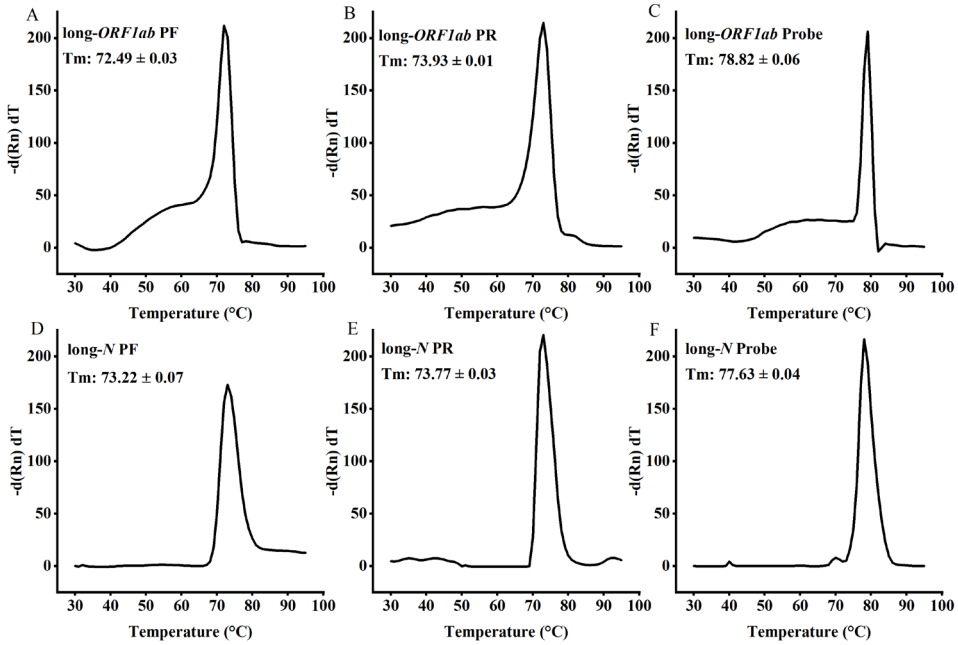

Figure 2. The melting curves of primes and probes that can work at the optimal elongation temperature of Taq DNA polymerase.

was about 1/6 of that of FQ Probe (Figure 4). Since the Spacer C3 group was cheaper than BHQ1 group, the FQS Probe was more cost-effective. And We designed the long-*N* FQS Probe and the long-*RP* FQS Probe according to the same principle.

3.3. The detection performance of the optimized primers and probe is excellent

The detection program of China CDC was taken as scheme 1 (CDC-*ORF1ab* PF, CDC-*ORF1ab* PR, CDC-*ORF1ab* Probe, CDC-*N* PF, CDC-*N* PR, CDC-*N* Probe, reaction program: reverse transcription in 55°C for 15 min followed by denaturation in 95°C for 30 s prior to undergoing 45 cycles of 95°C for 10 s and 60°C for 30 s). The primers and probe in scheme 1 were replaced with the optimized primers and probe, and the elongation temperature was changed from 60°C to 70°C, thus generating scheme 2 (long-*ORF1ab* PF, long-*ORF1ab* PR and long-*ORF1ab* FQS Probe, long-*N* PF, long-*N* PR and long-*N* FQS Probe, reaction program: reverse transcription in 55°C for 15 min followed by denaturation in 95°C for 30 s prior to undergoing 45 cycles of 95°C for 10 s and 70°C for 30 s). The limit of detection of scheme 2 was 500 copies/ml, which was consistent with that of scheme 1 (Table 2). Then a serial of PCR was carried out to reduce the elongation time at the elongation temperature of 70°C. Under four template concentrations, the Ct value of detection target remained unchanged when the elongation time was reduced from 30 s to 2 s. The Ct value increased when the elongation time was shorter than 2 s (Figure 5), indicating that the limit had been reached. In this case (Scheme 3), the limit of detection can still reach 500 copies/mL.

In terms of specificity, when the template concentration was 10^7 copies/mL, the Ct values of FAM channel and VIC channel were 23.29 and 23.37, respectively. When SARS-CoV, MERS-CoV, HCoV-

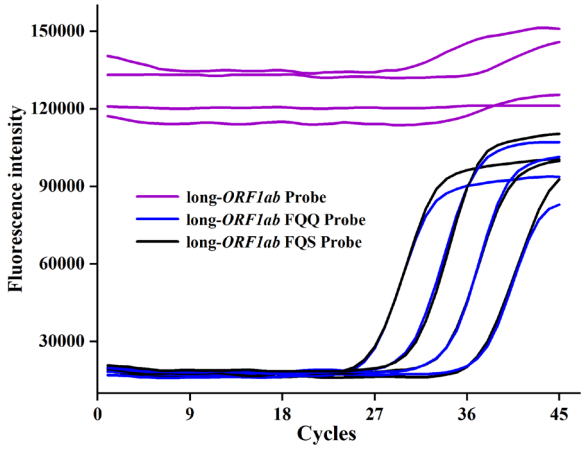

Figure 4. The raw data of RT-qPCR under four template concentrations using FQ, FQQ and FQS probes, respectively.

Table 2. Positive rate for schemes at different template concentrations

Schemes	Sample concentrations (copies/ml)				
	1000	700	500	400	300
Scheme 1	100%	100%	95%	85%	75%
Scheme 2	100%	100%	95%	80%	75%
Scheme 3	100%	100%	95%	85%	70%
Scheme 4	100%	100%	95%	75%	65%

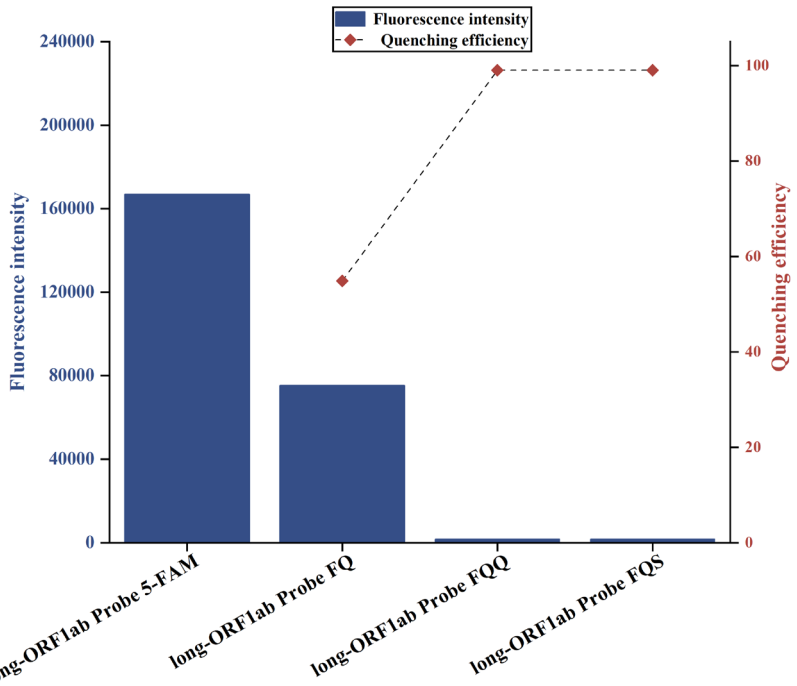

Figure 3. Fluorescence intensity of probes with different labeling types.

229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1 were used as templates, only SARS-CoV was amplified by the two detection channels, and the Ct values of FAM channel and VIC channel were 26.76 and 25.69, respectively. Amplification does not occur for other five HCoVs. Since the SARS virus has not been identified in the world since 2003, we thought that the specificity of scheme 3 was excellent.

These data indicated that Taq DNA polymerase do have a faster extension rate at 70°C in RT-qPCR reaction, and the time consumption was shortened from 74 min (Scheme 1) to 48 min (Scheme 3), which was 35.14% shorter than the China CDC standard procedure (Figure 6).

3.4. Optimization of reverse transcription and denaturation time to further reduced the time consumption

In order to further reduce the total time consumption, reverse transcription and denaturation time were also optimized based on scheme 3. Surprisingly, the limit of detection was still 500 copies/mL when the reverse transcription time was reduced from 15 min to 2 min and the denaturation time was reduced from 10 s to 1 s (scheme 4). The intra-group and inter-group coefficients of variation of the two detection channels were both less than 2.0%, indicating that scheme 4 had good repeatability (Table 3). In terms of detection time, the PCR amplification time and the total detection time of scheme 4 were 24 min and 26 min, respectively, which were 58.97% and 64.86% shorter than scheme 1 (Figure 6).

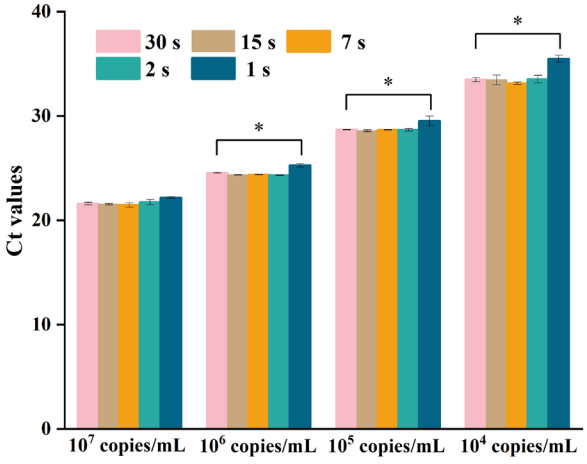

Figure 5. The RT-qPCR results under four template concentrations amplifying at 70°C for 30 s, 70°C for 15 s, 70°C for 7 s, 70°C for 2 s, 70°C for 1 s, respectively.

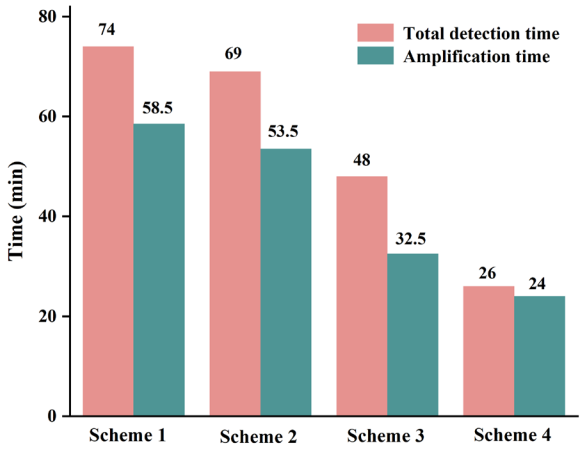

Figure 6. The total detection time and PCR amplification time of four schemes.

4. Discussion

RT-qPCR has long been used to detect a variety of pathogens and viruses (*16-18*). Rapid, accurate and low cost virus detection methods are critical for pandemic containment (*19*). On the one hand, rapid virus detection enables rapid tracking of primary contacts, prolongs therapeutic window and supports targeted treatment (*20*). On the other hand, it helps to interrupt the spread of the disease in the shortest time and at the lowest cost (*21*). In order to reduce time consumption, scientists have done a lot of work to improve the Taq DNA polymerase used in RT-qPCR: improving its reverse transcription activity for one-step detection (*22,23*); simplifying the Taq DNA polymerase by deleting unnecessary regions; improving the binding rate of Taq DNA polymerase to primer or template by combining Taq DNA polymerase with protein functional domain (Sso7d or Topo V) which can interact with DNA chain in the form of fusion protein (*24,25*); performing site-directed mutations and modifications to increase its elongation rate (*26*). There are also ways to increase the rate of temperature change by using special instruments and smaller reaction systems, such as droplet digital PCR (ddPCR), but this reduces the detection stability and throughput (*27*). Isothermal amplification has been viewed as a very successful method of diagnosis with high speed and sensitivity and no need for precise temperature control instruments (*28,29*), but most of the isothermal amplification systems include a complex enzyme system and special primers and probe, so have a high technical requirement (*11,30*).

In this research, by improving the Tm values of primers and probe, Taq DNA polymerase can be made to work at its optimal elongation temperature. This improvement combines with the optimization of the reverse transcription and denaturation time, finally reducing the detection time to 26 min on the premise that the limit of detection remained unchanged. The most important advantage of our strategy is this method can be implemented on a common RT-PCR instrument which has been popularized all over the world. Other methods, however, require updating instruments and thus may

Table 3. Repeatability analysis of primer pairs under different template concentrations

	Template concentration (copies/mL)	Intra-group experiments		Inter-group experiments	
		Ct Value (mean ± SD)	CV	Ct Value (mean ± SD)	CV
FAM channel	10^6	27.89 ± 0.17	0.61%	27.92 ± 0.19	0.68%
	10^5	31.73 ± 0.21	0.66%	31.81 ± 0.25	0.97%
	10^4	35.39 ± 0.36	1.02%	35.50 ± 0.34	0.96%
VIC channel	10^6	27.71 ± 0.12	0.43%	27.93 ± 0.37	1.32%
	10^5	31.83 ± 0.23	0.72%	31.93 ± 0.22	0.69%
	10^4	35.25 ± 0.33	0.94%	35.58 ± 0.36	1.01%

cause heavy financial burden. A critical preanalytical step for real-time PCR assays, as well as any assay in which nucleic acid is analyzed, is nucleic acid extraction. Currently, in order to conserve human resources, all testing institutions utilize automatic nucleic acid extractors. And the extraction time is about 20-30 min. The optimized fast RT-qPCR system and nucleic acid extraction time can complete the detection within about an hour, greatly improving the detection efficiency.

Acknowledgements

We thank Zhifeng Li, Jing Zhu and Jinyao Qu at Analysis & Testing Center of SKLMT (State Key laboratory of Microbial Technology, Shandong University) for assistance in use of qTOWER3 instrument for RT-qPCR assays.

Funding: This work was supported by the Shandong Provincial Youth Innovation Team Development Plan of Universities (No. 2022KJ194), Jining Key Research and Development Program (No. 2022YXNS158), Shandong Provincial Key Research and Development Program (No. 2020CXGC011305), Health Science and Technology Development Program of Shandong Province (No. 202201050463), Taishan Scholars Project of Shandong Province (No. tsqn202103186) and the Innovation Project of Shandong Academy of Medical Sciences.

Conflict of Interest: We have filed a patent application on the design and use of the primers.

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Received October 24, 2022; Revised December 18, 2022; Accepted February 18, 2023.

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Released online in J-STAGE as advance publication February 25, 2023.