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Development of a low-cost multi-channel nucleic acid detection PCR instrument and clinical detection application of COVID-19

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HIGHLIGHTS

- A more time-saving and cheaper two-channel real-time quantitative PCR instrument.
- Optimize the control algorithm to improve the control accuracy.
- Clinical verification was carried out on the P3 laboratory equipment in the hospital.
- More convenient to carry.

GRAPHICAL ABSTRACT
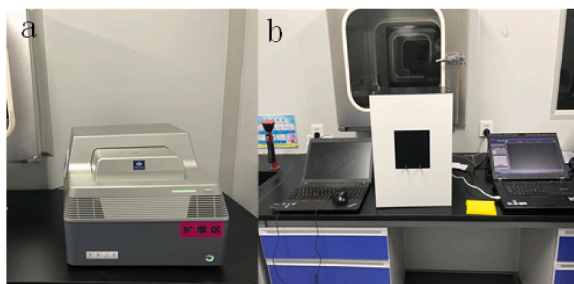
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ABSTRACT

Since the COVID-19 outbreak at the end of December 2019, a variety of novel Coronavirus nucleic acid detection methods have been proposed at home and abroad. Because of the disadvantages of most existing PCR instruments on the market such as long reaction time and high cost, this study developed a more timesaving and cheaper two-channel real-time quantitative PCR instrument. In this instrument, a PCR system combining a thermal cycle system and real-time fluorescence quantitative technology was designed. The software system and data processing, optical system, thermal cycle module, and hardware module of the PCR instrument were studied. The low-cost, portable real-time quantitative PCR system has been validated with consistent results compared to Bio-rad CFX Connect. At the same time, the same samples were used for the contract experiment with the hospital instrument, and the amplification result was better than the existing instrument in the hospital.

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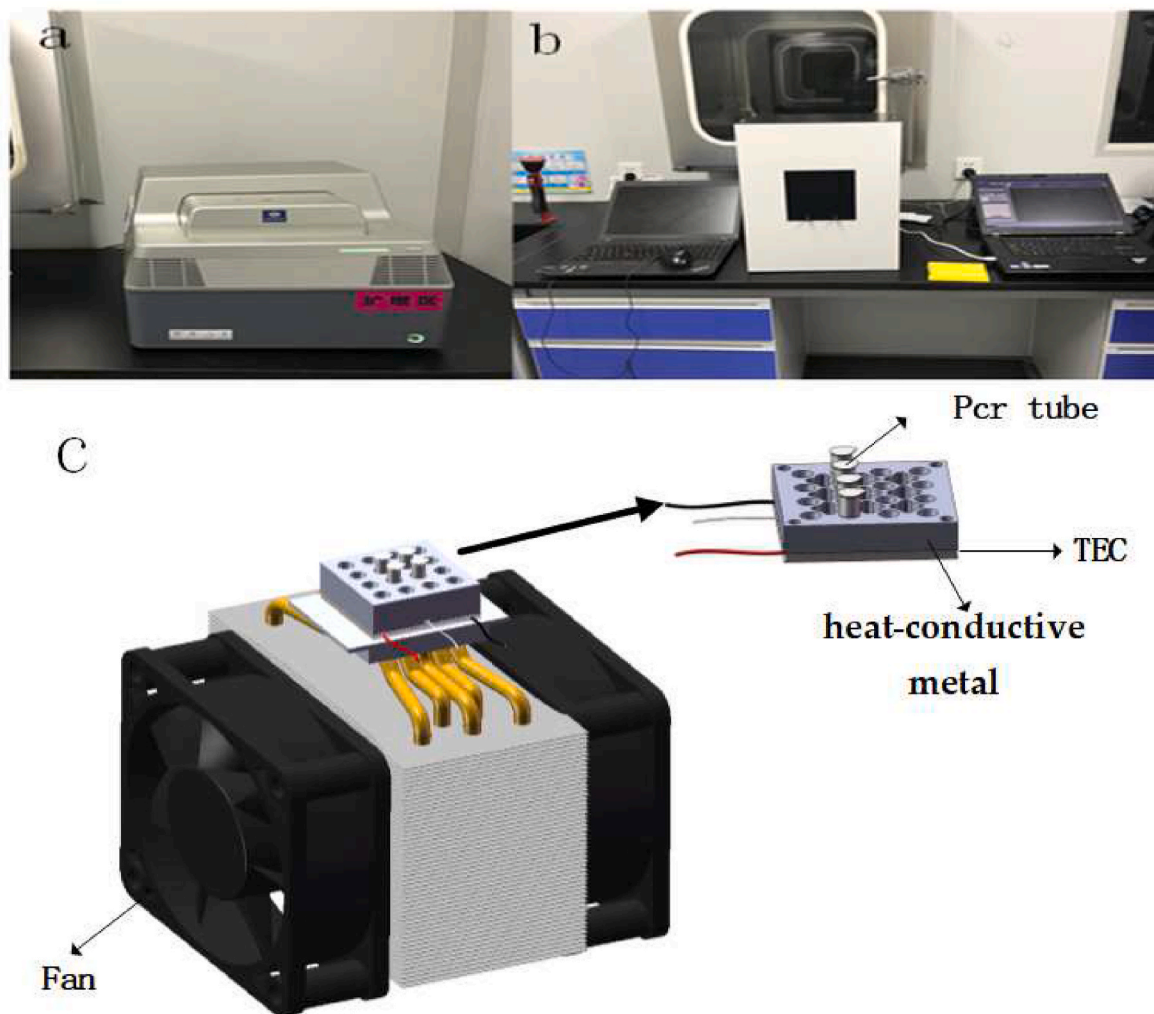Analytica Chimica Acta 1229 (2022) 340338

Fig. 1. Comparison between self-developed equipment and commercial equipment and introduction diagram of key heating components of self-developed equipmen (a) The photo showed the commercial qPCR equipment. (b) The image showed the homemade dual-channel qPCR instrument.(c) Temperature circulation system in self-made equipment.

1. Introduction

The instrument can realize real-time fluorescence quantitative detection and has significant application value in the biological field, especially in virus detection. The principle of PCR technology is similar to the natural replication process of DNA. Under the action of various enzymes, double-stranded DNA is denatured into single-stranded DNA. According to the complementary base pairing rules, new DNA can be reproduced from single-stranded DNA by using DNA polymerase, which can realize DNA amplification in vitro [1–4]. PCR consists of three basic reaction steps: denaturation - annealing - extension.

(1) Template DNA denaturation: template double-stranded DNA amplified by PCR or double-stranded DNA is dissociated into a pair of single-stranded DNA when being heated to about 93 °C after a while, which is easy to be combined with primer preparation for the next round of reaction;

(2) Annealing (refolding) template DNA and primers: Template DNA denatured during single-stranded heating, and the temperature drops to about 55 °C. The primers then bind to the template single-stranded DNA under the complementary base pairing rules;

(3) Extended primer coupling: DNA template and primer coupling can develop a new semi-reserved replication chain

complementarity according to the complementary base pairing rules template, during which DNA strand took dNTP as reaction products while took semi-conserved replication material and target sequence as the template. Under the action of Taq, DNA polymerase cycles catalyzed the multiplications of specific DNA through the three basic steps above [5–7].

At present, PCR apparatus mainly includes conventional PCR, gradient PCR, in situ PCR, and real-time quantitative PCR (qPCR) [8–11], while conventional PCR and real-time quantitative PCR are widely used. Quantitative fluorescence PCR technology was proposed by Higuchi in 1992 [12] when EB (ethidium bromide) was used as the fluorescent dye, and fluorescence excitation and detection device was installed based on the common PCR instrument. In the following development, ABI introduced the world's first fluorescence quantitative PCR instrument in 1996, whose real-time DNA DETECTION technology expanded the application field of traditional PCR. In 1998, Saiki et al. initiated RT-PCR technology, namely reverse transcription-polymerase chain reaction [13,14]. mRNA was first reversely transcribed into cDNA, and then PCR amplification and electrophoresis were performed to obtain the results to be detected, which could be used to determine the intensity of gene expression and identify whether the transcribed sequence had mutations.

Real-time quantitative fluorescence PCR technology (qPCR) was

Table 1
Primer and probe sequences.

Primer	Sequences (5′ to 3′)	5′ and 3′ double label modification
ORF1ab-F	CCCTGTGGGTTTTACACTTAA	
ORF1ab-R	ACGATTGTGCATCAGCTGA	
ORF1ab-P	CCGTCTGCGGTATGTGGAAAGGTTATGG	5′-FAM,3′-BHQ1
N–F	GGGGAACTTCTCCTGCTAGAAT	
N-R	CAGACATTTTGCTCTCAAGCTG	
N–P	TTGCTGCTGCTTGACAGATT	5′-VIC,3′-BHQ1

used in this experiment. RT-qPCR technology combines RT-PCR technology with qPCR to reverse-transcribed mRNA into cDNA and then amplified. At the same time, insertion dyes or fluorescent probes were used to make the amplified DNA have certain fluorescence. In combination with the relationship between fluorescence signal intensity and amplification increment, the number of replications of target DNA can be detected in real-time during amplification [15–19].

Since the emergence of COVID-19 in 2019, the epidemic has affected more than 100 countries around the world [20]. Up to August 13, 2021, there have been 206148237 confirmed COVID-19 cases and 4,342,587 cumulative deaths worldwide. The detection of novel coronavirus is an important part of controlling the epidemic. Therefore, it is imperative to develop a test instrument that is easy to operate and quick to measure [21,22].

In this study, aiming at the limitations of existing instruments such as high cost and poor accuracy, a new fluorescence detection system was developed by using real-time quantitative fluorescence PCR (qPCR) technology. To reduce the cost, the hardware system is restructured to achieve a much lower cost than the same type of commercial instruments, while optimizing the design of the control program algorithm and improving the control accuracy and operation speed to achieve higher accuracy and faster response speed. To verify its accuracy through experiments, clinical verification was carried out on the P3 laboratory equipment in the hospital, and consistent results were obtained.

2. Methods

2.1. Thermocycling system

The function of the thermocycling system was realized by using a thermoelectric cooler (TEC; ATE1-TC-127-8ASH, Analog Technologies) on a heat pipe radiator, where an aluminum well reaction plate was placed above, and the contact surface between them was evenly coated with a layer of one-component room temperature vulcanized thermally conductive silica gel (Youwo Industrial materials Co., Ltd, Dongguan, China) to enhance heat transfer and fix each device. A PT1000 thermistor (Hayashi Denko, Japan) was installed in the small hole on the side of the good reaction plate and connected to the temperature controller to obtain feedback temperature. The device and thermocycling system are shown in Fig. 1. The temperature controller adjusted the voltage and direction of the TEC, so that the temperature of the good reaction plate can be programmed to meet the temperature conditions required by PCR.

2.2. Reagents

The PCR reagent contained Abstart One-Step RT-PCR Mix (Diamond, Sangon Biotech, Shanghai, China), 0.5 μM forward and reverse primers, 0.25 μM probe, and PCR template. The gene of ORF1ab and N was inserted into RNA, which was further used as the PCR target, The primer sequences were as Table 1. We also used the novel coronavirus 2019-

nCoV nucleic acid detection kit (Sansure Biotech, Hunan, China) to test the negative and positive controls. The 20 μl PCR reagent was packed in a 0.1 ml flat cap thin-wall PCR tube and covered with 20 μl mineral oil (M8410, Sigma-Aldrich, MO) to prevent evaporation of the reagent during heating.

2.3. Fluorescence detection system

The fluorescence imaging system consists of two lasers (Anford, Taizhu Tec, Shenzhen, China) with a plane homogenization sheet, a Complementary Metal-Oxide-Semiconductor (CMOS) camera (E3ISPM20000KPA, Kuy Nice, China) with a Machine Vision Lens (V3528-MPY, Computar, Tokyo, Japan), and two narrow band-pass filters (535 nm ± 22.5 nm, 574 nm ± 15 nm, Rayan Tech, Changchun, China). The excitation light from the Lasers and two lasers respectively emitted light with wavelengths of 480 nm and 520 nm to excite the fluorescent probes in the PCR reagents. Two filters were embedded on a 3D-printed turntable. The turntable was driven by a stepping motor to switch the filters. The fluorescent signal of the reagents was captured by the CMOS through the filter and was transformed into pictures, while the fluorescence intensity of the reagents was measured by analyzing the gray value of the picture.

3. Results and discussion

We respectively tested the dual-channel real-time fluorescence PCR system, detected the ORF1ab and N genes in the new coronavirus, and modified the probes with FAM and VIC fluorophores. We used commercial test kits, and our reaction reagents to test the negative and positive controls, synthetic RNA, serum quality control products, etc. Comparing with CFX connect and LineGene 9600 Plus (Bioer Technology, Hangzhou, China), etc., we used the same reagents for horizontal comparison to verify system performance.

3.1. RT-qPCR detection progress

Put the configured reagents into the PCR tubes and insert them into the hole of the aluminum well plate, the device was connected to the computer through the USB port. Turning on the power of the device, the computer software set the reaction parameters, and the temperature controller drove the TEC to reach the set temperature through serial communication.

First, maintained the temperature at 42 °C for 30 min to allow RNA to complete the reverse transcription process to form DNA strand, then maintained at 95 °C for 5 min to complete the preheating process to inactivate the reverse transcriptase, and completed 40 cycles, at 95 °C for 15s, 60 °C for the 30s, respectively. At the end of each cycle, firstly turn on the 480 nm laser to activate the reagent in the PCR tube, and the fluorescence emitted by the reagent passes through the 535 nm filter was captured by CMOS as a fluorescence image. Then turn off the 480 nm laser and turn on the 520 nm laser. The motor drove the filter wheel to rotate, and the filter in front of the lens was switched to 574 nm. CMOS captured the fluorescence signal. The process was repeated every cycle to obtain 40 fluorescence images. The software extracted the gray value of the area where the PCR tube was located, deducting the background noise, and performing data fitting automatically. At the same time, it can obtain the amplification curve of the samples, and calculate the cycle thresholds (Ct) automatically.

3.2. The fluorescence of gene amplification analysis

The gradient-diluted synthetic RNA was used as a template to detect the ORF1ab gene, and the TaqMan probe was modified with the VIC fluorophore. The excitation wavelength and emission wavelengths were 520 nm and 570 nm respectively. The same set of reagents was divided into two equal parts and was placed apart into the self-made PCR system
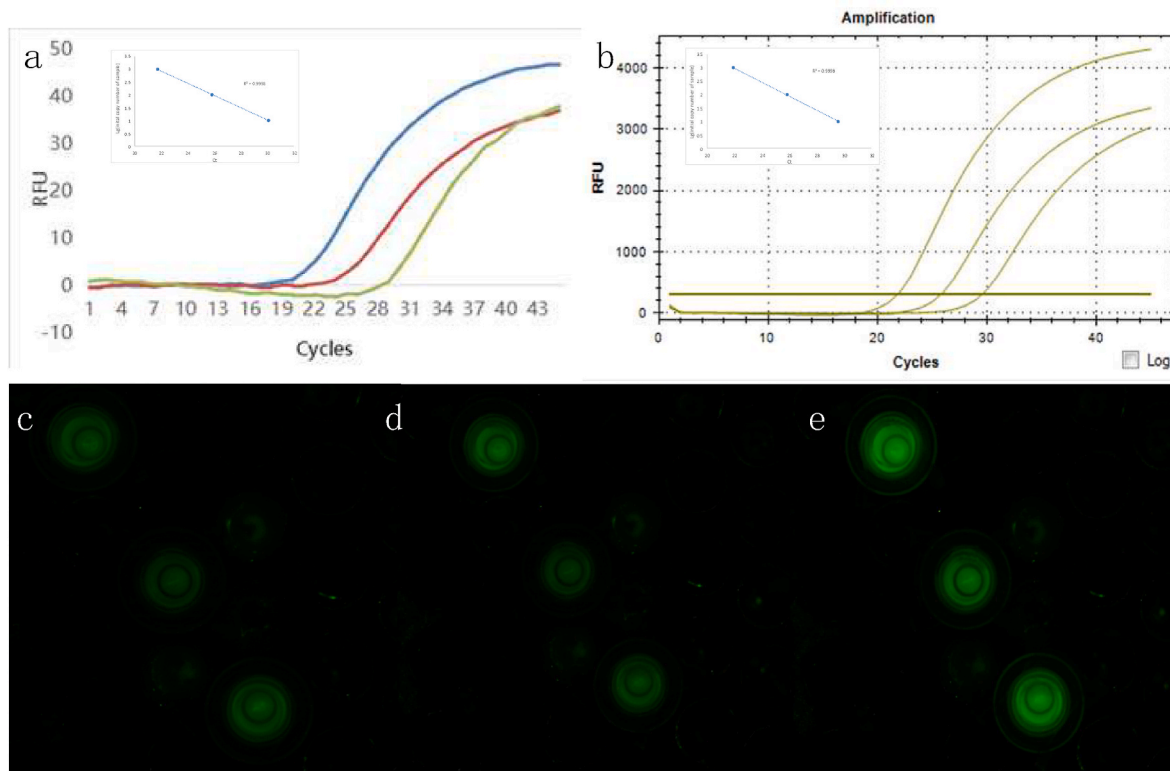
Fig. 2. Comparison of amplification curves of self-developed equipment and commercial equipment (a) The Amplification curves of three templates from the qPCR system. (b) Amplification curves from the commercial real-time PCR amplification system (Bio-Rad CFX connect). (c–e) The real-time images of the fluorescence from the CMOS when the number of cycles was 1, 25, and 45 were shown.
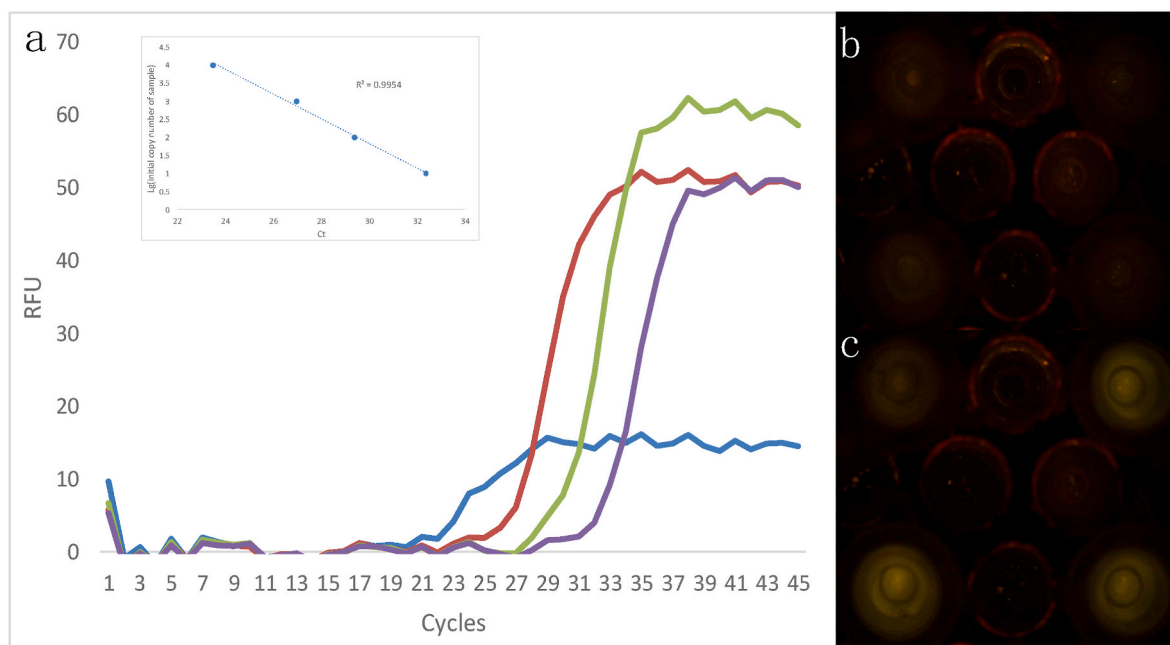

Fig. 3. Amplification curve of concentration gradient samples of self-developed equipment (a) Serial dilution of PCR amplification curves from the qPCR system. (b, c) The real-time images of the fluorescence from the CMOS when the number of cycles was 1 and 45 were shown.

and commercial instrument for testing. Fig. 3 ab showed the fluorescence amplification curves, the Ct values for 3 orders of concentration gradient were calculated to be 21.73, 25.84, and 30.13 for the self-made PCR system, and 21.92, 25.87, and 29.62 for the commercial cycler. the R2 correlation coefficient between them was calculated to be 0.9998 by

linear regression analysis, which proved the accuracy of the results detected by the qPCR device. Fig. 2c-e showed the fluorescence images at 15, 25, and 45 cycles. The fluorescence intensity of the reagents gradually increased, and the fluorescence intensity of different concentrations of reagents varied in time. The higher the template
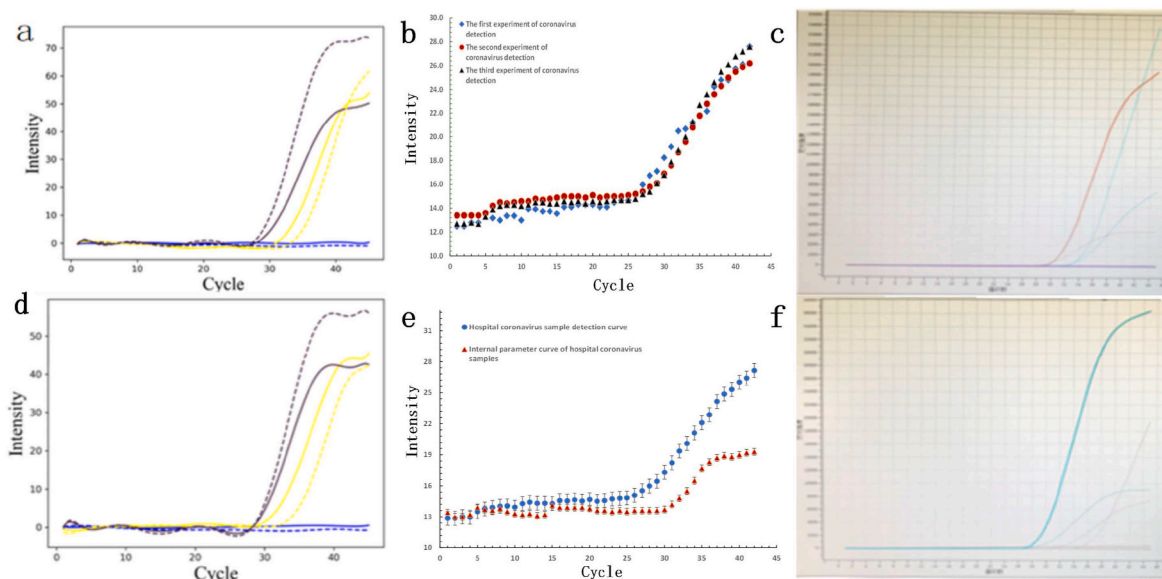
Fig. 4. Comparison of results of self-developed equipment and commercial equipment of China's top three hospitals on sample testing (a, d) PCR amplification curves obtained from the fluorescence images of the dual-channel qPCR system. (b,e) Multi channel fluorescence signal detection results of self-developed equipment, amplification curves of internal reference detection channels and sample detection channels. (c, f) The amplification curve was obtained from the commercial real-time PCR amplification system (Bioer, LineGene 9600 Plus).

concentration raised, the earlier the fluorescence increased. The stability of the system can be proved by comparing the results of the same sample between different devices.

3.3. Quantitative detection of the concentration gradient

The real-time PCR results shown in Fig. 3 demonstrated the amplification results for 4 ordered serially diluted reference genes by a homemade qPCR system. N was embedded in a plasmid as a sample, VIC fluorophore modified TaqMan probe, 520 nm laser-excited reagent, CMOS captured 570 nm wavelength fluorescence, 45 times of data were collected, and the amplification curve (Fig. 3a) was drawn from this. Fig. 3 b, c showed the fluorescence images of the 1 cycle and the 45 cycles, respectively. After PCR, the fluorescence intensity of the reagents was enhanced. The cycle thresholds (Ct) for the real-time PCR reactions were determined using a threshold detection method. The Ct values for 4 orders of magnitude concentration from 1×10^4–1×10^1 copies/µL were calculated to be 23.48, 26.97, 29.39,and 32.38. The correlation coefficient R^2 of the standard curve was calculated to be 0.9954. The RT-qPCR system had good quantitative accuracy.

3.4. Results of RT-qPCR

We tested the same reagents on the dual-channel PCR system and commercial equipment, compared the results, and performed two repeated experiments. In Fig. 4, the black was a positive sample 1, the yellow was a positive sample 2, the blue was a negative sample, the solid line was the FAM fluorescence channel (ORF1ab), and the dotted line was the VIC fluorescence channel (N). In commercial equipment, orange and light blue represented different samples. In the first repeated experiment, the commercial instrument sample 1 FAM channel Ct value was 32.5, VIC channel was 31.28, sample 2 FAM channel Ct value was 34.12, VIC channel was 35.24, and our equipment detected the sample 1 FAM channel Ct value was 29.7, VIC channel was 28.83, sample 2 FAM channel Ct value was 32.67, VIC channel was 34.21. In the second repeated experiment, commercial instrument sample 1 FAM channel Ct value was 30.4, VIC channel was 29.78, sample 2 FAM channel Ct value was 34.39, VIC channel was 35.77, while our equipment detected that the Ct value of the sample 1 FAM channel was 28.54, the VIC channel

was 27.88, the Ct value of the sample 2 FAM channel was 30.54, and the channel was 32.37. In repeated experiments, the dual-channel fluorescence PCR system could detect the fluorescence intensity changes of the two fluorescent channels and obtain detection results similar to those of commercial equipment. At the same time, we used the dual channel PCR system again to detect the coronavirus samples provided by the hospital. The results are shown in Fig. 4B and E. Fig. 4B shows the repeated experiments of the new crown samples and the multiple experimental verification of the self-developed equipment. It can be seen that the results of each experiment are close, and the CT values of the three experiments are about 25 cycles. Fig. 4E shows the results of dual detection channels in the dual channel PCR system. The blue curve is the sample data of the N site of the new crown, and the red curve is the sample data curve of the internal reference of the new crown. Fam channel used for N-site detection and Cy5 channel used for internal reference detection. It can be seen from the figure that the CT value of the N-site sample of the new crown is 25, and the CT value of the internal parameter is 29, which meets the current detection standard of the new crown. The perfect operation of the system can be confirmed.

4. Conclusion

Real-time fluorescence PCR is an important molecular diagnostic method as well as an important part of the epidemic prevention and control process. This paper proposed a dual-channel fluorescence PCR system, which controlled the temperature cycling system and the fluorescence detection system uniformly through a PC. A kind of automatic control program was designed to control the temperature change, switching the excitation light sources and the filters, while capturing the fluorescence image, which can analyze the data according to the images and draw the fluorescence change curve. For a variety of reaction reagent systems and samples, the RT-qPCR detection was successfully realized using this system, which proved the versatility and stability of the system. Based on this system, more fluorescence channels can be achieved through simple upgrades. And under the advantage of lower cost, there will be great potential for the large-scale application and promotion of PCR detection technology in the future.

CRediT authorship contribution statement

Shuang Ma: Validation. **Kangning Wang:** Data curation, Formal analysis, Writing – review & editing. **Yangyang Jiang:** Writing – original draft. **Yu Guo:** Writing – original draft, Writing – review & editing. **Yipeng Zhang:** Writing – review & editing. **YingJun Gao:** Writing – review & editing. **Wenming Wu:** Conceptualization, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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