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Application of automatic feedback photographing by portable smartphone in PCR

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ABSTRACT

The present PCR instrument is based on the Peltier semiconductor to achieve temperature cycling, but continuous photographing is not allowed and the self-contained timing software was seriously lagging. Therefore, from the perspective of photoelectric control, the conventional PCR machine was combined with a smartphone and PC, and an optical and electrical feedback automatic fluorescence detection system was designed to achieve quantitative real-time PCR. Through the script, camera timing was controlled by comparing the changes on the screen in the adjacent time, and automatic photo feedback is realized. This experiment has proven that this method can greatly improve the accuracy of the time to photograph and clearness of the photos. The smartphone connected to a computer installed on the PCR can be used after debugging. Compared with quantitative real-time PCR, conventional PCR is more cost-effective on the basis of detection requirements.

1. Introduction

The PCR machine has obvious application value in the fields of medicine, biology and so on [1–4]. The main working principle of the PCR machine is similar to the natural replication process of DNA, that is, double-stranded DNA can be denatured into a single-strand DNA under the action of various enzymes. Under the action of DNA polymerase, the single-strand DNA can be copied from itself into a new one, according to the principle of complementary base pairing. The PCR consists of three basic reaction steps: denaturation-annealing-extension. (1) Denaturation of the template DNA: The template double-stranded DNA or double-stranded DNA amplified by PCR is dissociated to a single strand after heating to approximately 93 °C for a certain period of time, making it convenient to be combined with the primer in preparation for the next round of reactions; (2) Annealing (refolding) of the template DNA and the primer: The template DNA is denatured into a single strand by heating, and the temperature drops to approximately 55 °C. Then, the primer combines to the template of the single strand DNA through the principle of complementary base pairing; (3) Extension of the primer: The conjugate of the DNA template and primer can develop a new semi-reserved replication strand complementary to the template DNA strand according to the principle of complementary base pairing and semi-conservative replication, with dNTP as the reaction material and the target sequence as a template, under the action of Taq DNA polymerase. By repeating the cycle-denaturation-anneal-extension process, more "semi-reserved replication strands" can be obtained, and this new strand can be used as a template for the next cycle. It takes a short time to complete a cycle, and the gene that needs to be amplified can be amplified several million times in 2–3 hours [5]. At present, the PCR machine mainly includes conventional PCR, gradient PCR, in situ PCR, and quantitative real-time PCR (qPCR). Among these, the most widely used are conventional PCR and qPCR [5–8]. Compared with conventional PCR, qPCR includes the fluorescence signal acquisition system and computer analysis processing system. The primers added in the amplification are marked with fluorescein, and the primers and fluorescent probes are combined with the template at the same time [9–13]. The results of the amplification are collected in real-time by the fluorescence signal acquisition system, while the quantitative real-time output results are obtained by the computer analysis processing system. However, the cost shown in Table 1 of qPCR on the market is usually approximately 10 times the cost of conventional PCR. Therefore, it is of great significance to realize qPCR by simply modifying conventional PCR.

At present, ubiquitous smartphone technology for portable chemistry and biometrics is evolving [14–22], a large number of researchers have carried out studies on qPCR through fluorescence detection using a smartphone or camera [2,21,23–31]. It can be observed that combining a smartphone and personal computer with a conventional PCR

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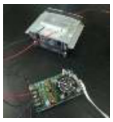
Table 1
Comparison of different PCR.

Brand	device	size	detection window	Detection method	Quantification	Cost (¥)
TEC		Small	No	N/A	No	550
Junsi		Large	No	agarose gel electrophoresis	No	2700
LifeECO		Large	No	agarose gel electrophoresis	No	27,000
BIO-RAD (CFX Connect)		Large	Photodiode	fluorescence detection	Quantitative	300000 -600000
Homemade (Modification of the above PCR)		N/A (Depending on the basic equipment)	Smartphone or camera	fluorescence detection	Quantitative	1000-10000 (Depending on the price of the phone or camera)

machine is a feasible way to achieve quantitative real-time PCR.

However, there are certain difficulties in the process of achieving qPCR with the combination of fluorescence detection by smartphone and a conventional PCR machine. Since the long-term illumination of light would cause the deterioration and discoloration of fluorescent dyes, the qPCR requires the signal detection mode of interval photographing. That is, photographing is normally performed only during the denaturation or extension stage. In most of the documents, photographs are manually taken at regular time periods, making it very inconvenient [22,23,31]. When the software installed on the phone was used to control the taking of photographs at regular time periods, it is found that the actual picture taking interval of the camera should be the software setting time plus the response time and exposure time of the camera [23,32,33]. Although the exposure time of the camera can be set, the response time of the camera varies with different working conditions [22]. Hence, it can be observed that the actual interval for photographing is difficult to control.

From the perspective of photoelectric control, the present study combined a conventional PCR machine with a smartphone and personal computer, and designed an optical and electrical feedback automatic fluorescence detection system to achieve the quantitative real-time PCR.

2. Methods

2.1. Thermocycling system

The instrument mainly includes three parts: thermocycling system, temperature feedback system, and optical feedback system (Fig. 1).

The function of the thermocycling system was realized using a thermoelectric cooler (TEC; ATE1-TC-127-8ASH, Analog Technologies) and a temperature controller (TCM1031). An aluminum plate with a NTC thermistor (MF55) was inserted on top of the TEC mentioned above. A RTV silicone rubber was applied to that plate to achieve a uniform heat distribution. Then, several aluminum plates was placed in a circle as cooling fins, which was placed under the TEC with a radiator fan to help effectively promote heat conduction. An infrared (IR) camera (Fotric 220; ZXF Laboratory, TX, USA) worked as the feedback to check the temperature of the tubes with the reagent.

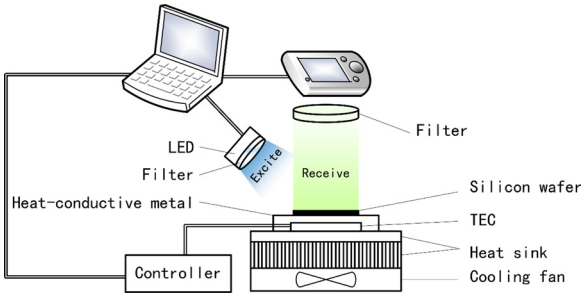


Fig. 1. Schematic drawing of the instrument.

2.2. Optical feedback system

2.2.1. Fluorescence imaging was performed using different brands of cameras and smartphones

The cameras and smartphones were chosen based on the gradient of its price and camera effects, in order to ensure good contrast among the experimental data. The smartphone was connected to the computer through the Mobile Assistant software installed on the computer. This allowed the picture captured by the phone lens to be displayed on the computer screen in real-time. The program script to control the photographing was pre-written in the computer. The fluorescence was excited at 470 nm through a LED array (XPE3W, Cree, NC), and filtered with a narrow band-pass filter (470–30 nm, Xintian Bori, Beijing, China). The LED array equipped beside the tube was periodically turned on/off by a control relay circuit. When photographing was required, the LED array would light up, and at the same time, generate a signal by illuminating the screen. Then, the program script receives that to control the smartphone with its lens covered using an emission filter (520–40 nm; Xintian Bori, Beijing, China) to press the shutter to take a photograph. ImageJ software was used to quantify the fluorescence intensity, and the data was graphed using Excel for systematic analysis.

The 360 Mobile Assistant software (mobile version; http://sj.360.cn/index.html) was installed on the smartphone, while the Quick Macro software (http://www.anjian.com/) and 360 Mobile Assistant software (personal computer version) were installed on the personal computer. The software used for this system was all free. The smartphone was

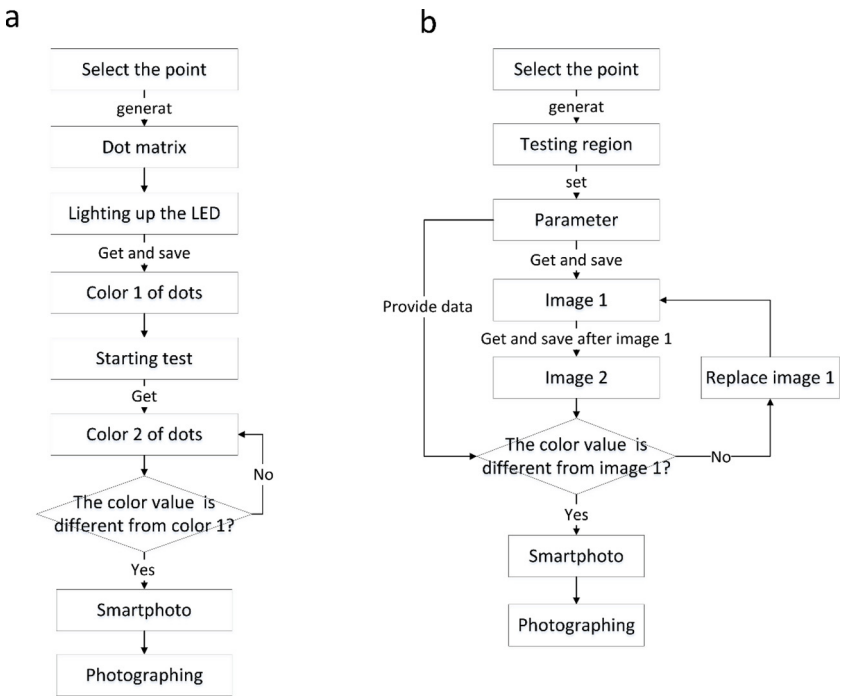


Fig. 2. (a) The flow chart of the pixel-point method to control the photographing operation. **(b)** The flow chart of the image area method to control the photographing operation.

connected to the personal computer via a USB cable. With the 360 Mobile Assistant software installed in the smartphone and personal computer, the smartphone screen was monitored and remotely controlled by the personal computer. That is, the camera photographing screen of the phone can be observed on the PC screen in real-time. By running the script written using the Quick Macro software, optical feedback automatic photographing could be achieved.

2.2.2. Two methods were proposed to achieve the automatic photo feedback of the script

The first method (Fig. 2a) is that the script performs the photographing operation according to whether the color of a single pixel in the monitoring screen changes. Before the cycle starts, it is necessary to artificially collect a pixel with a significant color change in the monitoring screen when the LED switch state changes. After the cycle starts, the script would continuously detect whether the color of the selected pixel changes. When the color of the selected pixel significantly changes (in this case, the LED turns on), the shutter button is automatically pressed to complete the photographing operation. The main disadvantage of this method is that the stability is not good, and when the color value of the selected point does not significantly change, the camera will not take the photograph.

In order to improve the stability of automatic feedback photographing, a second method was proposed (Fig. 2b). In the second method, the script continuously detects no longer than a single pixel point, except for a rectangular area with larger changes, when the LED turns on. After the cycle starts, the script continuously saves and compares the images of the selected area. When the difference between two adjacent saved images becomes obvious (the LED light turns on), the script automatically completes the photographing operation. This method can change the sensitivity of automatic photographing through the plug-in of the Quick Macro software, achieving the parameter control of the automatic photographing system. These experimental results show that the second method is better than the first method. In the end, the second method was adopted to achieve the automatic photographing feedback.

2.3. Reagents

The performance of the system was verified by comparing the performance between the quantitative real-time PCR (CFX Connect; Bio Rad, CA) and the combined system of smartphone and conventional PCR listed in Table 1. The PCR reagent contained 1× Premix Ex Taq HS, 1× EvaGreen Dye, 0.5 μM of forward and reverse primers, and the PCR template. The primer sequences were as Table 2. These genes were inserted into a pUC57-Kan plasmid vector (Genewiz, Suzhou, China) by recombinase, and was used as the PCR target.

2.4. Melt curve analysis

It is of great necessity to analyze the melt curve after each reaction, in order to check the specificity of the PCR assay, since fluorochrome binds to all double-stranded DNAs.

The specific product and other products have different melt curve. With the degeneration of the two-strand DNA, the fluorochrome would return free, and decrease the fluorescence signal. The target melt curve should have a single peak, and the comparison of the annealing temperature of the peak position with that of the specific product can determine whether the expanded production is the target product.

In the system, the melt temperature is controlled by a programmable temperature controller. The temperature of the melting rises from 65°C to 95°C, rising steadily with a temperature gradient of 0.5°C

Table 2
The primer sequences of genes.

genes	primer sequences
Rubella virus (RUBV)	F: 5'-ATTGTTATGTATGAGCGGTGAA-3' R: 5'-TTGTAAAGCCCTATGAGTGAGC-3'
H7N9	F: 5'-CAGACAATCCCCGACCGAA-3' R: 5'-GCCAAGTGTTAGCCCCATCC-3'
PGEM-3ZF (+) 1	F: 5'-CCAGTCGGGAAACCTGTCGTGCC-3' R: 5'-GTGAGCGAGGAAGCGGAAGAGCG-3'
PGEM-3ZF (+) 2	F: 5'-AATACTTTTGCGAGGAGACCCA-3' R: 5' –ATTGCCATCTACACCAACCCA-3'

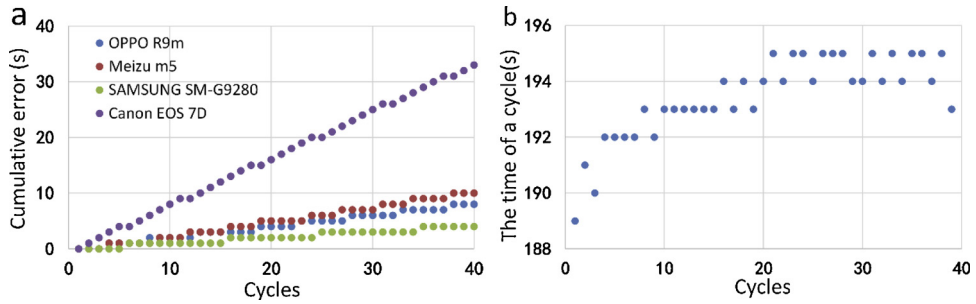


Fig. 3. (a) The cumulative error curves for different brands of smartphones and cameras; **(b)** The change on the time required to perform one cycle with the total number of cycles for conventional PCR.

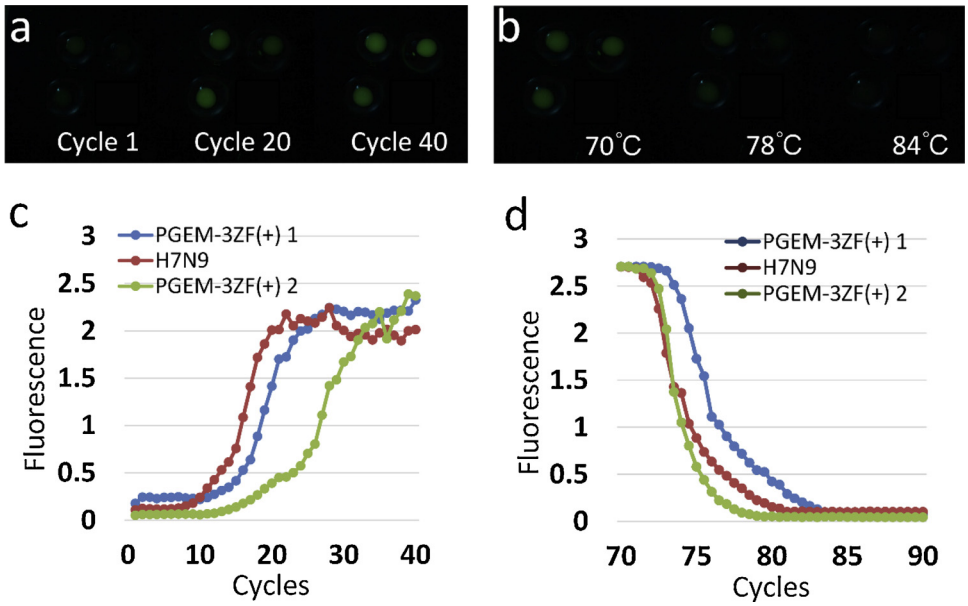


Fig. 4. (a) The real-time image of the fluorescence from the smartphone when the number of cycles was 1, 20 and 40 is shown. **(b)** The real-time image of the fluorescence from the smartphone when the temperature was 70 °C, 78 °C and 84 °C is shown. **(c)** The Amplification curves of three templates from the combined system of smartphone and conventional PCR. **(d)** The melt curves of three templates from the combined system of smartphone and conventional PCR.

and staying for 5 s at each gradient. At this point, the program controls the smartphone to take a photo. The above scheme is compiled into controller readable information and uploaded to the controller.

3. Results and discussion

3.1. Error analysis

The reaction time of different brands of cameras and smartphones is not the same. At the same time, the exposure time is different. Therefore, it is very important to reasonably choose the brand of smartphone and camera. The cumulative error of three different models of smartphones and one camera in the amplification process (Fig. 3a) reveals the influence of the brand on photography, and the difference in clearness of the photography between the camera and smartphone. Overall, all the equipment's errors increased with the number of cycles. Among these, the increase in error for the Cannon EOS 7D was rapid, while for the Meizu m5, OPPO R9Am and Samsung SM-G9280, this was slow. Furthermore, the error for the Samsung SM-G9280 was the smallest (the closest one to the actual requirements). However, the camera had a low delay, when compared to that, and the delay for the three smartphones was more serious. The difference among the delay curves of different smartphones show that the delay of each phone is not reproducible. For conventional PCR, the time of each cycle (Fig. 3b) also differed as the amplification progressed, and the error was approximately five seconds. Hence, conventional PCR is not stable in practical application. Compared to the smartphone's error, which ranged within 5–10 seconds, the using a smartphone to take photographs of fluorescence can replace qPCR.

For PCR, the amount of product obtained during the extension phase was very important for later analysis. Hence, the requirements for the timing of photographing were relatively high (60 °C was selected). If the photographing time was too early, the extension was not over, and the brightness of the fluorescence was darker than the actual situation. If the photographing time was too late, the PCR temperature would increase, some of the double-strand DNAs would be dissociated, and the fluorescence brightness captured would be darker than the actual situation. This problem was solved by using photoelectric transformation to make time for smartphones to be able to more accurately take photographs. After many tests, the temperature at the time of photographing was stable at 60 °C.

3.2. Comparison of fluorescence detection results between the application of automatic feedback photographing using the portable smartphone system and the second generation PCR

3.2.1. Multi-sample analysis

Another group of experiments set the template type to a variable, and the device used the same smartphone to prove that other templates could be detected using the same method, and that these images could be clearly identified. Three samples of PGEM-3ZF (+) 1, H7N9 and PGEM-3ZF (+) 2 were placed in the same region of the PCR, in order to ensure these were amplified under the same conditions. The fluorescence shown in Fig. 4a show the rapid amplification of the sample during the amplification period, and the fluorescence rapidly brightened in 40 cycles. However, at the time of melting (Fig. 4b), the

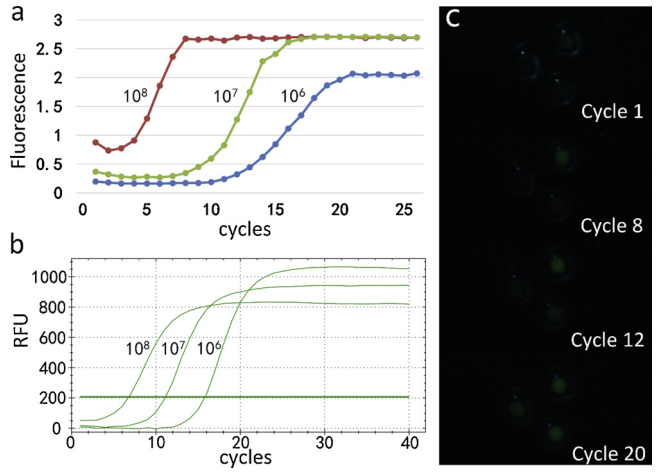


Fig. 5. (a) Amplification curves for the combined system of smartphone and conventional PCR. **(b)** Amplification curves for the real-time PCR amplification system. **(c)** The real-time image of the fluorescence from the smartphone when the number of cycles was 1, 8, 12 and 20.

fluorescence faded again. The amplification curve shown in Fig. 4c reflects the amplification of three samples. Although the fluctuation of the curve was relatively large, the curves of each sample could be clearly distinguished. The melt curve analysis was run to testify the specificity of the product, as shown in Fig. 4d. The S-shape curve revealed that the product was specific, and the curve is highly recognizable, which meant that smartphones were suitable for photographing with different templates.

3.2.2. Template gradient analysis

Fig. 5 shows the amplification results of different concentrations of the same template (PGEM-3ZF (+) 1) in the qPCR and the combined system of smartphone and conventional PCR. Compared with real-time fluorescent PCR, there was no additional software involved in processing the data obtained from conventional PCR. Therefore, the amplification curve (Fig. 5a) was not as smooth as that for real-time fluorescence PCR (Fig. 5b). However, it was clear and allowed for a higher concentration to be obtained. Firstly, we select the fluorescence brightness of the second to eighth cycles of one of the curves, then calculate the standard deviation of these values. Finally, the fluorescence threshold is obtained by adding the average value of fluorescence brightness with ten times standard deviation. It was necessary to perform different degrees of translation downward, and the number of cycles corresponding to the intersection of the fluorescence thresholds was the Ct value, since the RFU in the amplification curve gained by real-time fluorescent PCR was the relative value after moving the curves. The Ct values of the real-time fluorescent PCR were 6.8, 11.15, and 15.83, and the Ct values calculated by the combined system were 5.9, 11.5, and 15.3. Furthermore, the less the initial number of cycles that the template began to amplify and the overall condition of the curve proves that the amplification curves obtained through these two methods is approximate. The real-time image of the fluorescence of three concentrations obtained from the smartphone (Fig. 5c) was also clearer. The results above show that the combined smartphone and conventional PCR for detecting fluorescence can have a clearer response to different concentrations of DNA amplification of the same template.

The template was replaced with RUBV, and the concentration was unchanged. The amplification curves and melt curves shown in Fig. 6 were obtained. Compared with Fig. 5, the detection results of the template used this time were closer to that of the qPCR. The amplification curve obtained from the smartphone (Fig. 6a) was numerically different from that obtained from the qPCR (Fig. 6c). This phenomenon could be easily observed by comparing the melt curve obtained from the smartphone (Fig. 6b) and that obtained from the qPCR (Fig. 6d). However, the trend of amplification was the same, and it was easy to distinguish the specificity of the product. The Ct values given by qPCR were 11.53, 15.26 and 17.5, and the Ct values calculated by the combined system were 11.8, 16 and 18.4. In order to avoid the contingency of a single experiment, two repetitive experiments were performed, and the standard deviation was calculated by using the data of three experiments, and the results were showed in Fig. 6a. The amplification image (Fig. 6e) clearly shows the change in fluorescence of the sample from the beginning to the end of the cycle. Fig. 6f shows the change in fluorescence of the product after 65 °C. Upon reaching 95 °C, the product was almost completely dissociated.

4. Conclusion

This paper provides a combination system based on TEC temperature control module and smart phone, which can realize the function of qPCR at very low cost. More importantly, automatic feedback

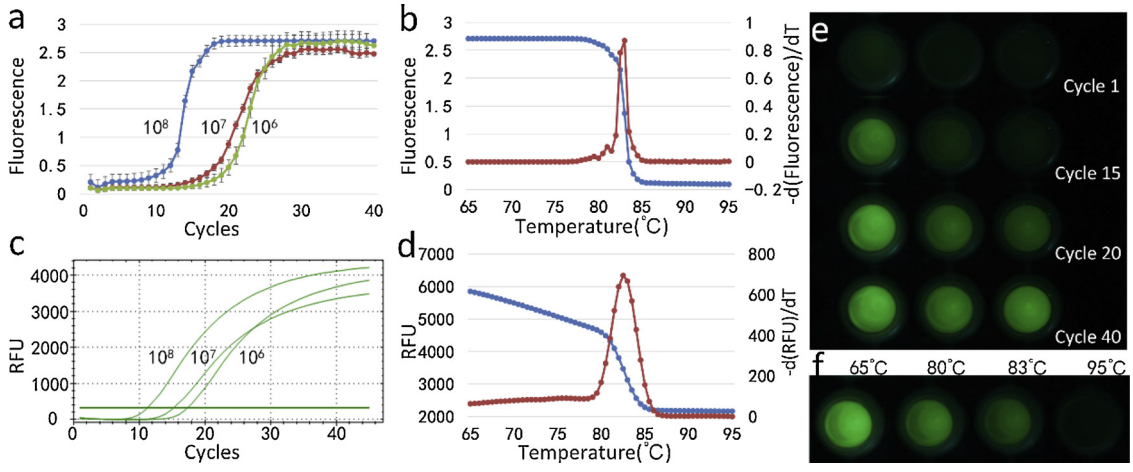


Fig. 6. (a) The amplification curves from the combined system of smartphone and conventional PCR are shown. **(b)** The melt curve analysis of the template with a concentration of 10^6 copies/ul obtained from the combined system of smartphone and conventional PCR, with the first derivative change in fluorescence intensity as a function of temperature, are shown. Meanwhile, only a single peak corresponding to the PCR product was observed. The amplicon is clean and specific. **(c)** The amplification curves from the real-time PCR amplification system are shown. **(d)** The melt curves from the real-time PCR amplification system are shown. **(e)** The real-time image of the fluorescence from the smartphone when the number of cycles was 1, 15, 20 and 40. **(f)** The real-time image of the fluorescence from the smartphone when the temperature was 65 °C, 80 °C, 83 °C and 95 °C.

photographing using a portable smartphone in PCR provides a method for transforming conventional PCR into expensive qPCR. This automatic feedback photographing system is easy to use and manufacture. The conventional PCR modified by this method would obtain the function of real-time fluorescence detection. The time interval for a smartphone to take photographs can be adjusted using a script installed on the computer. In the present, the structure, working principle and actual detection effect of the system are emphatically described. In addition, all experimental data show that it can replace the fluorescence detection system in qPCR to meet the needs of fluorescence detection in conventional PCR.

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Declaration of Competing Interest

The authors declare no competing financial interests.

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