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A new thermal cycling mechanism for effective polymerase chain reaction in microliter volumes

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Abstract This study presents a new thermal cycler using infrared (IR) heating and water impingement cooling for polymerase chain reaction (PCR) amplification of 10 μl samples in thin glass capillary tubes. The thermal cycling system can achieve a temperature ramping-up rate of 65 °C/s and a ramping-down rate of 80 °C/s. Two other cooling mechanisms, natural convection and forced air convection, can also be used in the present system to obtain a ramping-down rate of 2 °C/s and 6 °C/s, respectively. The amplification of the 439 fragment of hepatitis B virus (HBV) DNA was successful. The PCR amplified products were analyzed by agarose gel electrophoresis with ethidium bromide staining for visualization. A comparison of results of the amplification products at three different ramping-down rates was made and the rapid thermal cycling of the present system can run DNA required amplification in 29 min for 20 thermal cycles that is only 1/3 the time spent in the conventional PCR machine used in comparison.

1 Introduction

Amplification of specific DNA fragment in vitro using the polymerase chain reaction (PCR) requires a well controlled thermal cycling [1, 2, 3]. There are three stages in the PCR. The first stage is denaturation at which the sample is heated up from room temperature to denaturation temperature at approximately 90–95 °C. The high temperature results in a separation of the double strands of DNA into two single stranded DNAs. The second stage is called annealing at which the sample temperature drops from the denaturation temperature to annealing temperature at approximately 40–65 °C. At the annealing stage, added primers in the sample are paired with single stranded DNA through a Brownian motion in the solution and subsequent hydrogen-bonding. The final step is elongation (or extension) at which the sample temperature is heated up from the annealing temperature to the elongation temperature at approximately 72 °C. Since the primers already have a strong ionic bonding to the DNA template on the specific locations at the annealing stage, dNTPs are added complementarily to the DNA template. A complete temperature cycle consists of the temperature variations in the three stages in PCR. To obtain enough yield of products, the number of temperature cycles required depends on the type of DNA for amplification.

In a conventional PCR machine, thermal cycling is performed on a 10 μl sample in a microcentrifuge tube using a temperature controlled metal block. The major setback of this conventional thermal cycler is a long temperature lag in the sample behind the metal block. Consequently, amplification time for DNA products may usually take a few hours. To reduce the thermal cycling time, Wittwer and his co-workers constructed a rapid cycling system by introducing hot air to heat up the sample in thin capillary tubes [4]. In their system, the thermal cycling time for 20 cycles was reduced from 1–4 h in a metal block thermal cycler to 20 min. In addition, their system can obtain better specificity in amplification products with less transition time from denaturation to annealing temperatures.

A new thermal cycling system for the PCR on a 160 nL sample was introduced by Huhmer and Landers [5] by using a Tungsten lamp as a heating source and forced air convection as cooling mechanism. The sample was placed in a thin glass capillary tube. Compressed air was blown to the capillary tube for rapid cooling and infrared light emitted from the Tungsten lamp could provide rapid heating on the samples. With proper controls on the applied voltage to the lamp and an air flow rate, the sample temperature can be maintained at a stable level. Since the 160 nL sample has a low heat capacity, the temperature ramping-up rate for IR heating is 65 °C/s and the temperature ramping-down rate is 20 °C/s for forced air convection cooling in their system. Their amplification products were separated through capillary electrophoretic process and were detected with laser-induced fluorescence (CE-LIF). Their CE results showed that a faster temperature transition significantly improves efficiency of PCR

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in amplification of DNA and reduces formation of non-specific products.

In recent years, fabrication techniques were applied to integrate both PCR and CE functions on silicon or glass substrates to serve as a Lab-on-a-chip. The integrated systems for achieving efficient DNA amplification, capillary electrophoretic separation, and laser-induced fluorescence detection have been demonstrated by various groups [6–11]. For a monolithic integrated DNA system [11], PCR amplification was performed in a 0.28 μl chamber to which heating and cooling of thermal cycling were performed by a thin film heater and a nitrogen gas flow respectively. A 10 °C/s temperature ramping-down rate was achieved. A similar work was performed by Peltier [6] for PCR-based DNA analysis in a micro fabricated chip but his work employed thermoelectric devices as both heating and cooling sources. The PCR chamber was sandwiched between two thermoelectric devices and the temperature ramping-up and ramping-down rates were 2 °C/s and 3 to 4 °C/s respectively. These micro-fabricated biochips are better integrated and easier to operate than the present capillary glass tube system for DNA analysis. However, few works have been conducted to increase the efficiency of PCR amplification on these micro-fabricated biochips.

However, better efficiency in PCR amplification can be achieved by a short transition time from the denaturation step to the annealing step [12]. The slower the temperature transition, the higher the probability for the primer to bond with itself, the so-called primer dimer formation; or to bond with sequences in the DNA template that are not complimentary to the primer, the so-called nonspecific amplification. The aim of this study is to reduce the temperature transition time and improve the efficiency in PCR amplification. An IR radiation for heating combined with a water impingement for cooling resulted in a thermal cycle as short as 57 s and achieved successful PCR amplification of genomic DNA in 10 μL samples in the capillary glass tube.

2 Process development

2.1 Thermal cycling system

A schematic view of a thermal cycling system for PCR amplification is shown in Fig. 1a. This system consists of a Pentium-4 PC, a lamp, a UV cut filter, a water container, a water pump, two capillary glass tubes, and a plexiglass holder. As shown in Fig. 1b, two capillary glass tubes, one for testing and the other for temperature measurement, are placed side by side on the top of the holder. The capillary tube (Micro-hematocrit capillary tube, OXFORD, USA) is 7.5 cm in length, 1.1 mm in inner diameter and 1.4 mm in outer diameter. Both capillary tubes contain 10 μL sample for PCR amplification. Before filling the sample into the capillary tubes, the capillary tubes are sequentially cleaned with DI water; 99% acetone, 30% hydrogen peroxide, 96% sulfuric acid and 29% ammonium hydroxide. After the sample is filled into the capillary tubes, 3 μL mineral oil is added into each end of the capillary tubes. The capillary

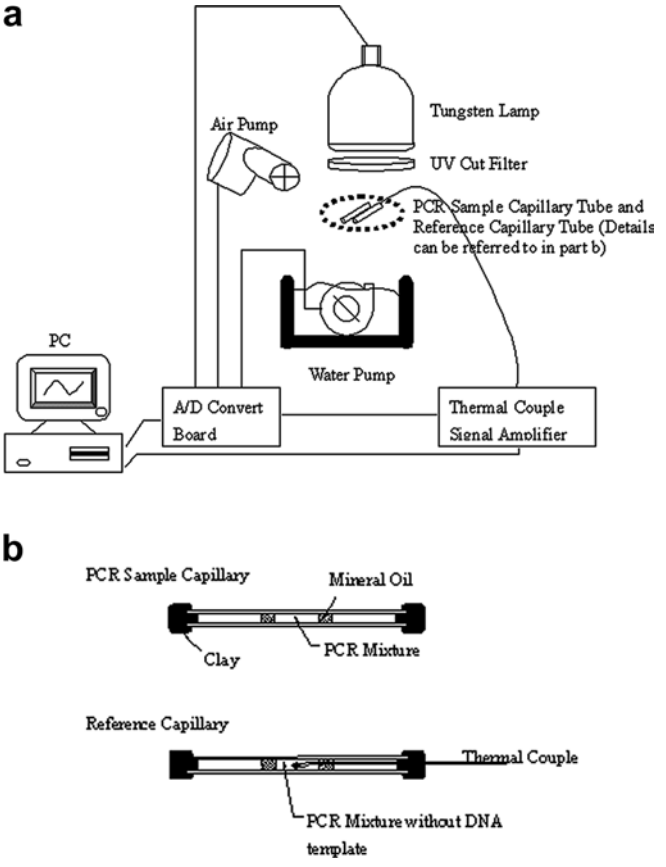

Fig. 1a, b. Schematic view of the thermal cycling system for PCR amplification. **a** Detailed setup, **b** magnified view of the capillary tube with 10 μL sample and the reference capillary tube with thermocouple for temperature measurement

tubes are then sealed with clay. Sample temperature response is measured from the sample in the reference capillary tube instead of that in the tested tube because the metal content of thermocouple can contaminate the DNA sample. A K type thermocouple with a 41 μV/°C sensitivity is installed in the sample of the reference capillary tube. The analog signals that are measured from the thermocouple are amplified by a linear amplifier (Aecl). The amplified temperature signals are then converted by a digital board (NuIPC Compact PCI DAQ Card, ADLINK, Taiwan) with a 12 bits resolution for temperature sampling and recording. The data acquisition rate of sample temperature is 20 Hz. A closed-loop control algorithm with sampled temperature signals are used to control the ramping rate of temperature and accurate dwelling temperature of thermal cycle. The controlled program is coded in Visual Basic Language. The measured temperature history of sample in the reference capillary tube is recorded in the PC. Tested sample in glass capillary is heated up by a Tungsten lamp (50 W Tungsten lamp, OSRAM, Germany). To study the effect of temperature ramping-down rate on DNA amplification, the samples with the same mixture are prepared for three separate runs that have different cooling mechanisms namely natural convection, forced convection by compressed air, and water impingement by mist ejected from a water pump. These

various cooling mechanisms can provide different ramping-down rate of the tested capillary tube.

2.2
Rapid thermal cycling

As shown in Fig. 1, the PCR sample is heated by the Tungsten lamp powered with an 110 V AC/DC transformer. The lamp intensity is modulated by a pulse width modulation through a solid-state relay. To prevent photolysis of the PCR sample, an optical long wave pass filter with a 400 nm transmission cutoff is installed between the Tungsten lamp and the capillary tubes. Emitted light from the Tungsten lamp passing through the UV cut filter is absorbed by the sample. Since the K type thermocouple is only installed in the middle of the reference capillary, there might be temperature difference between the sample and the reference tubes due to the small focusing area of Tungsten lamp. It is found that the temperature difference between the two capillary tubes is less than 0.1 °C by exchanging the positions of both tubes. The temperature maintenance and ramping rate can be controlled by switching from a PID control approach. In the theory, the control on lamp intensity through the PWM modulation can precisely maintain the sample temperature with a variation of ±0.4 °C at 94 °C in denaturation, a variation of ±0.2 °C at 56 °C in annealing and a variation of ±0.3 °C at 72 °C in elongation regardless of cooling methods used.

With the same IR radiation heating, the thermal cycling system can employ three different cooling mechanisms, namely natural convection cooling, forced air convection cooling, and water impingement cooling. In the natural convection mode, the Tungsten lamp was turned off and the capillary tubes are cooled down by natural convection. The temperature ramping down rate is at 2 °C/s. In the forced air convection mode, compressed air at room temperature is supplied from a 20 psi pressurized chamber to cool down the capillary tubes. Exiting from a nozzle, cooling air directly impinges on the capillary surface. The nozzle diameter is six times of the diameter of capillary tube for assuring uniform cooling on the capillary tube surface. A 6 °C/s ramping down rate can be achieved. With the water impingement mode, water mist is supplied from a water pump that is submerged in the water chamber. The stream of water mist directly impinges on the capillary tube surface. Without proper treatment on the capillary surface, the formation of water film on the capillary tubes becomes inevitable. Consequently, it will add thermal mass on the tested sample. The time to heat up the sample to setting temperatures will also go up. Therefore, the outer surface of the capillary tubes is sprayed with hydrophobic coating 30-μm thick silicon compound (Unick Chemical Corp, UH-102) to prevent the formation of water film. The fastest ramping down rate can be up to 80 °C /s with water impingement cooling.

2.3
PCR amplification in conventional thermal cycling instrument

The DNA template amplified in the 0.6 ml microcentrifuge tube in the compared conventional PCR thermal cycler using the temperature controlled metal block (Biometra TRIO-Thermoblock™, Biotron, Germany) is used for comparison with the PCR amplified products from the proposed thermal cycling system. For HBV DNA amplification, it contains 20 cycles of 60 s of denaturation at 94 °C, 30 s of annealing at 56 °C and 60 s of elongation at 72 °C. A first cycle requires an extra 5 min of denaturation time at 94 °C and the final cycle requires an extra 5 min of elongation time at 72 °C. For comparison, the present thermal cycler has the same setting temperatures and dwelling period at denaturation, annealing, and elongation stages as those in the compared conventional PCR machine except that the transition time from one designated temperature to the other is different between the conventional PCR machine and the present one. In addition, the temperature ramping up rate is kept as the same as IR radiation heating but the ramping down rate is different among the three cooling mechanisms.

2.4
PCR using HBV dimer DNA template

For testing the proposed thermal cycler, the amplification fragment tested is 200 pg/μL HBV dimer DNA template. Each 10-μL sample is composed of 100 pg HBV, 6.5 μL ddH_2O, 1x reaction buffer (KT 10x, Ab Peptides Inc., USA), 20 μM each dNTP, 2.5 units Taq polymerase (KlenTaq1™ DNA Polymerase, Ab Peptides Inc., USA), 25 ng ST-3s (5′-AGGA-CCCC-TGCT-CGTG- TTAC-3′;181–200) primer and SB-4a (5′-AGAT- GATG-GGAT-GGGA-ATAC- 3′; 629–600) primer to amplify the 439 bp length specific DNA fragment.

2.5
Analysis of the PCR product by slab gel electrophoresis

The amplified products generated by PCR in the compared conventional conventional thermal cycler and the present thermal cycler are characterized by slab gel electrophoresis. PCR products and DNA standards are separated with a 2% agrose gel(GX 04004, GenAgarose, Genaxis™ Biotechnology). Electrophoresis of amplified products is performed using a Mini Gel Migration Trough (Mupid-2, COSMO BIO Co., LTD.) at an applied voltage of 10 V/cm for 20 min in 1x TAE buffer (Ultra Pure Grade, Amresco Inc., USA). The DNA is visualized with ethidium bromide staining. The staining dye is contained in 6x gel-loading buffers: 0.25% bromophenol blue and 40% (w/v) sucrose in H_2O. Through electrophoresis, the observation of DNA fragments is conducted by a short wavelength UV transilluminator (Mighty Bright, UVTM, Hoefer).

3
Results and discussion

Figure 2 shows the agarose gel electrophoretic results of DNA amplified products by the present thermal cycler at three different ramping-down rates and those by the conventional thermal cycler but at the same setting on denaturation temperature at 94 °C for 60 s, annealing temperature at 56 °C for 30 s and elongation temperature at 72 °C for 60 s for 20 thermal cycles. The first cycle preheats the sample at 94 °C for an extra 5 min and the final cycle keeps the sample at 72 °C for another extra

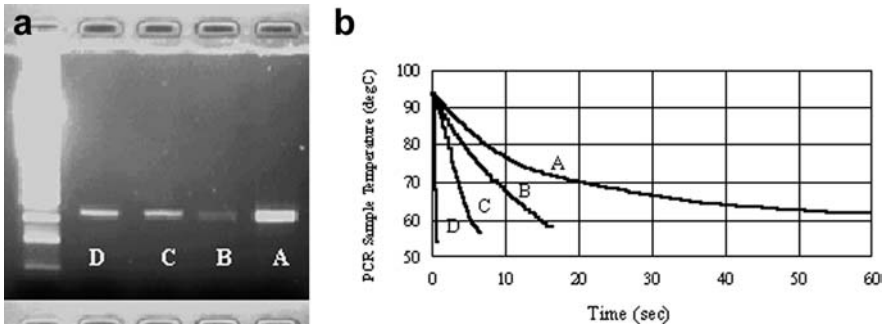

Fig. 2a, b. Effect of sample temperature-time profile on amplified products. **a** Agarose gel electrophoretic results of amplified products for 20 thermal cycles at four different tested conditions, namely commercial PCR machine (Lane A), natural convection (Lane B), forced air convection (Lane C), and water impingement (Lane D). The leftmost lane is for DNA fragment standard. **b** Corresponding transition times for different tested conditions

5 min before the sample is cooled down to room temperature. However, the transition time from one setting temperature to the other in each thermal cycle is different among the four tested conditions, as shown in Fig. 2b. The agarose gel electrophoretic result shown by lane A in Fig. 2a is obtained from the amplified products by the conventional PCR machine. Due to a large heat capacity of heating block, the conventional PCR machine has the low temperature ramping-down rate at around 0.8 °C/s. Agarose gel electrophoretic results represented by lanes B, C, and D are from the amplified products by the present thermal cycler with natural convection cooling, forced air convection cooling, and water impingement cooling, respectively. The temperature ramping-down rates are 2 °C/s for natural convection, 6 °C/s for forced convection and 80 °C/s for water impingement. As shown in Fig. 2a, the gel electrophorectic bands of amplified products from the present thermal cycler are all narrower but darker than that from the conventional PCR machine. It indicates the conventional PCR machine has the best product yield. However, the gel electrophoretic band from lane B to D were with narrow band and could have better resolution on the agarose gel electrophoretic results. The band B to D were brighter and brighter while the temperature ramping-down rate gets greater and the transition time gets shorter and that shows a better product yield obtained from a shorter transition time from the denaturation temperature to annealing temperature. Based on the results shown in Fig 3, water impingement cooling is has the best yield among the three cooling mechanisms tested in the present thermal cycler. Unless otherwise stated, water impingement cooling is the method used in thermal cycler presented in the following section.

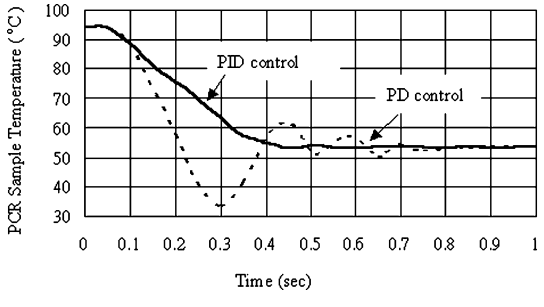

Fig. 3. Effect of control approach on temperature transition from denaturation temperature to annealing temperature

Since the short transition time in PCR thermal cycle plays a key role in the DNA amplification efficiency, the present thermal cycler is tested to obtain the shortest transition time from denaturation to annealing temperatures. The highest ramping-down rate with water impingement cooling can be achieved by switching the temperature control algorithm from a PID control approach to a PD control approach. As shown in Fig 3, the temperature ramping-down rate can reach a value of 200 °C/s. For amplified results shown in Fig 2, the PID control approach is used for all three cooling mechanisms. In comparison with the PID control approach, there is no integral error correction in temperature response in the PD control approach. In the PD control approach, the water pump is on and the heating lamp is off when the sample temperature is higher than the setting temperature. On the opposite, the water pump is off and the heating lamp is on as the sample temperature is lower than the setting temperature. However, in the PID control approach, the heating lamp is always on, but the lighting intensity is adjusted according to the integral error correction at the cooling stage. As shown in Fig 3, the PCR sample temperature can drop form denaturation temperature to annealing temperature in 0.13 s at the PD control mode. However, the PD control approach causes a much larger temperature fluctuation than the PID control approach. Such a large undershooting in sample temperature fails to produce specific DNA amplification.

Figure 4a shows a rapid thermal cycling with water impingement cooling for 20 cycles on PCR amplification of sample by the present thermal cycler. Note that this rapid thermal cycling still requires an extra 5 min to preheat the sample at 94 °C before the first cycle and another 5 min at 72 °C after the last cycle. The dwelling times in each thermal cycle are 10 s at denaturation, 10 s at annealing, and 20 s at elongation. A successful amplification on HBV dimer DNA fragment is carried out in 29 min for 20 thermal cycles. The PCR thermal cycling time by the present thermal cycler is only one-third the time by the conventional PCR machine. The agarose gel electrophoretic results of DNA amplified products that are visualized from ethidium bromide staining are shown in Fig 5. The electrophoretic band by the proposed rapid thermal cycling is much narrower than that by the conventional PCR machine. It indicates that the band intensity of conventional PCR machine strongly has a much better product yield on DNA amplification than the present rapid thermal cycling. However, better yield is not always

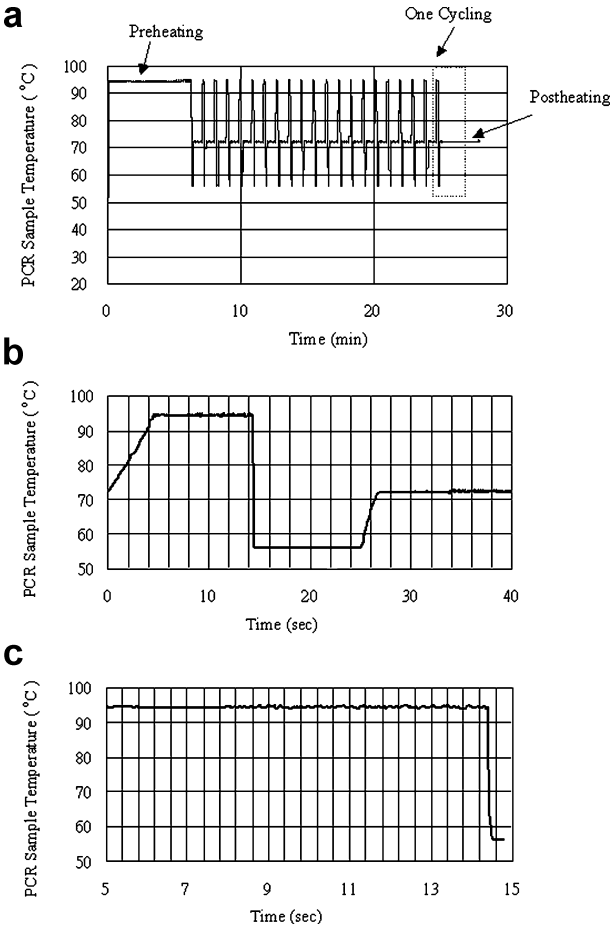

Fig. 4a–c. Rapid PCR thermal cycling using IR radiation source. **a** 20 cycles consisting of 10 s at 94 °C, 10 s at 56 °C, and 20 s at 72 °C, **b** one cycle with consisting time as **a**, **c** expanded scale representation of temperature profile of denaturation stage

desirable. Relatively low yield is required when consensus primers are used to detect a group with the sequence to be amplified is not precisely known. The narrow band with good resolution can be produced by rapid thermal cycling with the same protocol performed on the conventional PCR machine.

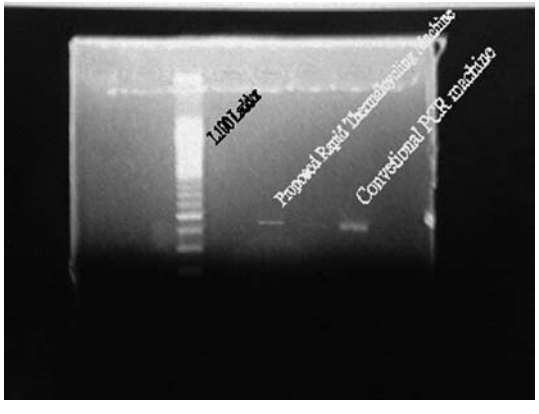

Fig. 5. Agarose gel electrophoretic results of proposed thermal cycler and commercial PCR machine with cycling times shown in Fig. 4

4 Conclusion

A rapid thermal cycler is proposed in this study for PCR amplification of DNA by employing IR radiation for heating and water impingement for cooling 10 μL sample in capillary glass tube. With water impingement cooling and PID control approach, an 80 °C/s temperature ramping-down rate of PCR sample in capillary tube can be achieved while the temperature in the PCR thermal cycling drops from denaturation temperature to annealing temperature. Such a high ramping-down rate of 80 °C/s in the present thermal cycler is about two orders of magnitude faster than that of 0.8 °C/s in the compared conventional PCR thermal cycler. Our present thermal cycler has a lower product yield than the conventional PCR machine. However, the present thermal system can produce narrow band for better agarose gel electrophoretic results reading since the high yield rate is not always desirable. Among all three different cooling mechanisms tested in the present thermal cycler, water impingement cooling can obtain higher product yield than natural convection cooling and forced air convection cooling.

An extremely fast temperature ramping-down rate of 200 °C/s can be obtained by using the PD control approach while the thermal cycling is conducted on 10 μL DNA sample in capillary glass tube with water impingement cooling. However, despite of the fastest ramping-down rate obtained in the present thermal cycler, the undershooting problem in annealing temperature during the temperature transition from denaturation to annealing temperature prevents any successful DNA amplification.The present thermal cycling system using IR heating and water impingement cooling showed the extreme fast PCR thermal cycling temperature transition in the microliter volume capillary. Based on the PCR ingredient and protocol developed for the conventional PCR thermal cycling machine using temperature -controlled metal, the DNA yield rate is still lower by the present system. However, the narrow and sharp band yielded by the present system can offer better resolution of agarose electrophoretic results and the present system can run DNA amplification in 29 min for 20 thermal cycles that is only 1/3 the time spent in the compared conventional PCR machine.

References

1. **Mullis KB; Ferre F; Gibbs RA** (1994) The Polymerase Chain Reaction, Birkhauser, Boston
2. **Innis MA; Gelfand DH; Shinsky JJ; White TJ** (1990) PCR Protocols: A Guide to Methods and Applications. Academic Press: San Diego
3. **Eeles RA; Stamps AC** (1993) Polymerase Chain Reaction (PCR): The Technique and Its Applications. R. G. Landes Co: Austin, TX
4. **Swerdlow H; Iones BJ; Wittwer CT** (1997) Fully automated DNA reaction and analysis in a fluidic capillary instrument. Anal Chem 69: 848–855
5. **Huhmer AFR; Landers JP** (2000) Noncontact infrared-mediated thermocycling for effective polymerase chain reaction amplification of DNA in nanoliter volumes. Anal Chem 72: 5507–5512
6. **Khandurina J; McKnight TE; Jacobson SC; Waters LC; Foote RS; Ramsey JM** (2000) Integrated system for rapid PCR-based

DNA analysis in microfluidic devices. Anal Chem 72: 2995–3000
7. **Soper SA; Ford SM; Xu Y; Qi S; McWhorter S; Lassiter S; Patterson DD; Bruch RC** (1999) Nanoliter-scale sample preparation methods directly coupled to polymethylmethacrylate-based microchips and gel-filled capillaries for the analysis of oligonucleotides. J Chromatogr A 853: 107–120
8. **Robert TK** (1999) Bioanalytical applications of fast capillary electrophoresis. Analytica Chimica Acta 81: 163–180
9. **Guetens G; Cauwenberghe KV; Boeck GD; Maes R; Tjaden UR; der Greef JV; Highly M; van Oosterom AT; de Bruijn EA** (2000) Nanotechnology in bio/clinical analysis. J Chromat B 739: 139–150
10. **Guifeng J; Said A; Gregor O; William EL; Harison DJ** (2000) Red diode laser induced fluorescence detection with a confocal microscope on a microchip for capillary electrophoresis. Biosensors & Bioelectronics 14: 861–869
11. **Eric TL; Peter CS ; Richard AM** (2000) Monolithic integrated microfluidic DNA amplification and capillary electrophoresis analysis system. Sensors and Actuators B 63: 138–146
12. **Wittwer CT; Garling DJ** (1991) Rapid cycle DNA amplification: time and temperature optimization. BioTechniques 10: 76–83