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Capillary-based fully integrated and automated system for nanoliter polymerase chain reaction analysis directly from cheek cells

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Abstract

A miniaturized, integrated and automated system based on capillary fluidics has been developed for nanoliter DNA analysis directly from cheek cells. All steps for DNA analysis, including injecting aqueous reagents and DNA samples, mixing the solutions together, thermal cell lysis, polymerase chain reaction (PCR), transfer and injection of PCR product, separation, sizing and detection of those products are performed in a capillary-based integrated system. A small amount of cheek cells collected by a plastic toothpick is directly dissolved in the PCR cocktail in a plastic vial or mixed on-line with a small volume of PCR cocktail (125 nl) in the capillary. After thermal cell lysis and PCR in a microthermal cycler, the DNA fragments are mixed with DNA size standards and transferred to a micro-cross for injection and separation by capillary gel electrophoresis. Programmable syringe pumps, switching valves, multiposition and freeze–thaw valves are used for microfluidic control in the entire system. This work establishes the feasibility of performing all the steps of DNA analysis from real samples in a capillary-based nanoliter integrated system.

Keywords: Instrumentation; Automation; DNA

1. Introduction

DNA analysis using polymerase chain reaction (PCR) is one of the most widely practiced methods for biological and clinical research. Standard DNA analysis from real samples based on PCR in plastic vials and separation by slab gel electrophoresis (SGE) typically requires large reaction volumes (tens of microliters), many steps performed in different devices, and human intervention at several stages. All of these result in high cost for reagents, time, labor and supporting equipment. To address these problems, immense efforts have been made to develop various microchip- or capillary-based miniaturized, integrated and automated systems for PCR analysis.

The idea of miniaturization and integration of conventional analytical protocols on a microchip, i.e., the so-called micro-total analysis system (μ-TAS), has been suggested for nearly a decade [1,2]. In recent years, many developments and applications of chip-based microfluidics to DNA analysis have been reported. The early works on the integration of chemical reactions with analysis on a microchip device dealt with simple reactions, such as fluorescence-labeling [3] and DNA restriction digestion [4]. The functional integration of complex reaction, such as PCR amplification, clean-up and electrophoresis analysis in a microchip device was demonstrated in 1996. In that work [5], a hybrid device that carried out PCR amplification in a polypropylene tube heated by a silicon heater and electrophoretic sepa-

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ration in a glass channel was reported. While it established the feasibility of integrating two fundamentally different and important functions into a single device, that report did not address some important problems, such as preparation of real samples for PCR, on-chip injection and mixing of DNA-template solutions with PCR cocktails, and miniaturization of the reaction volume. Recently, more sophisticated microchip devices have been fabricated and tested that have greater complexity, and consequently, improved functionality. For example, a system that demonstrated the potential of integration was constructed for the analysis of nanoliters of DNA [6]. The compact system was capable of measuring nanoliter-size (120 nl) aqueous reagent and DNA-containing solutions, mixing the solution together, amplifying or digesting the DNA to form discrete products, and separating and detecting those products. Nevertheless, the system cannot be considered as a complete μ-TAS for identifying DNA, as the preparation of raw DNA-containing samples was not incorporated. In another recent work [7], attempt was made to perform all of the steps of PCR-based DNA analysis from real samples on a single microchip where cell lysis, multiplex PCR amplification, and electrophoretic separation can be executed sequentially. However, the method did not incorporate on-chip injection and mixing of DNA samples with the PCR reagents and the reaction volume (ca. 10 μl) was not miniaturized. Moreover, manual intervention was required to move the microchip into and out of the radiatively coupled Peltier thermal cycler assembly.

Other research groups explored the potential of capillary-based integrated systems for DNA analysis. We and others have demonstrated that an integrated DNA sequencer from DNA template to called bases can be achieved by the use of capillaries as chambers for cycle sequencing reaction, product purification, separation and detection [8–10]. We also developed a capillary-based multiplexed and integrated system for PCR-based DNA typing directly from whole blood [11]. Similar to what has been implemented on microchips, these capillary-based methods integrate several important steps (e.g., DNA amplification and analysis), miniaturize the basic components (e.g., separation by capillary gel electrophoresis, CGE, requiring only nanoliters of DNA sample), and hence effectively address several critical problems (e.g., low speed and high labor cost) of conventional systems for DNA analysis. However, the great potential of capillary-based μ-TAS for DNA analysis has yet to be fully exploited.

Clearly, a system that can perform all of the steps of DNA analysis from real sample to final results with consumption of sample and reagent at the nanoliter scale will provide significant advantages to the biochemist in terms of speed, cost and automation. Although a variety of partially miniaturized and integrated systems based on microchips or capillaries have been demonstrated for DNA analysis, the miniaturization and integration of all steps of DNA analysis from real samples is not trivial. The major hurdle is the extremely high complexity of the microfluidic system necessitated by the incorporation of miniaturized sample preparation prior to PCR and the coinjection of DNA standards.

Currently, most PCR-based DNA tests from real samples require a DNA extraction and purification step, as most polymerases used in PCR are inhibited by typical substances present in crude samples. Traditionally, DNA was extracted according to the phenol–chloroform method [12] or by ''salting-out'' rapid purification [13]. In recent years, many new DNA purification methods have been developed, such as spin column gel filtration, magnetic beads binding and elution, and membrane electro-elution [14–16]. Almost all these methods require relatively large sample and reagent volumes (tens of microliters), numerous steps of manipulations (e.g., addition of lysis reagents, washing, elution, centrifugation, etc.) with different devices (heater, centrifuge, etc.), and hence do not allow miniaturization and integration at the nanoliter scale. An alternative concept to the miniaturization and integration of pre-PCR sample preparation is by developing more tolerant PCR conditions which can be applied directly to raw biological samples. A number of protocols for direct PCR from clinical materials have been successfully performed in plastic tubes [17–21]. Recently, our group has successfully demonstrated the direct PCR of whole blood in a large bore capillary (360 μm I.D.) [11] with a reaction volume of tens of microliters. However, off-line sample pretreatment by heating was still necessary and on-line mixing of PCR reagents and DNA samples was not incorporated. In

order to reduce the reaction volume to the nanoliter scale, direct PCR should be performed in smaller bore capillaries (e.g., 75 μm I.D.), and on-line thermal cell lysis and mixing should also be incorporated. The obstacle to performing direct PCR in small I.D. capillaries is that the polymerase may be inhibited by not only substances present in crude sample but also the inner surface of the capillary due to the large surface-to-volume ratio.

Here we report a miniaturized and integrated system based on capillaries where all steps of PCR-based DNA analysis directly from real sample were performed at the nanoliter scale. The system features the elimination of pre-PCR sample preparation. The integrated steps include injection of nanoliter PCR cocktails and DNA-containing raw samples, mixing of solutions, thermal cell lysis to release DNA, mixing the PCR products with DNA sizing ladder, transfer and loading onto electrophoresis device, separation by CGE and detection by laser-induced fluorescence (LIF). The functionality of the system is demonstrated through PCR–CE analysis of human genomic DNA directly from cheek cells.

2. Experimental

2.1. Materials

Anhydrous sodium hydroxide (NaOH), fuming hydrochloric acid (HCl) and tris(hydroxymethyl)-aminomethane (THAM) were obtained from Fisher Scientific (Fairlawn, NJ, USA). Tris–HCl (pH 8.3) buffer used for conditioning the reaction capillary was prepared by mixing 25 m*M* THAM and 4 m*M* HCl in deionized water. 10× PCR reaction buffer (100 m*M* Tris–HCl, pH 8.3 at 25 or 30 mg $MgCl_2$, and 2.5 mg/ml bovine serum albumin, BSA), 10× dNTP mixture (2 m*M* of each dNTP) and enzyme dilution buffer (10 m*M* Tris–HCl, pH 8.3, 2.5 mg/ml BSA) were bought from Idaho Technology (Salt Lake City, UT, USA). Taq polymerase (5 unit/μl), male human genomic DNA and formamide were purchased from Promega (Madison, WI, USA). A 100-base pair (bp) DNA ladder was obtained from Life Technologies (Gaithersburg, MD, USA). The three primer sets used to amplify the target fragments of the Y chromosome of human genomic DNA are: 5′AGGCACTGGTCAGAATGAAG3′ and 5′AATGGAAAATACAGCTCCC3′ for 209-bp product, 5′GGTTATCATAGCCCACTATACTTTG3′ and 5′ATCTTTATTCCCTTTGTCTT-GCT3′ for 256-bp product, 5′TGGTAAACTCTACTTAGTTT-GCCTTT3′ and 5′CAGCGAATTAGATTTTCTT-GC3′ for 393-bp product. These primer sets were synthesized by the DNA facility at Iowa State University (Ames, IA, USA).

The 1× TBE buffer solution [89 m*M* tris(hydroxymethyl)aminomethane, 89 m*M* boric acid and 2 m*M* ethylenediaminetetraacetic acid (EDTA)] was prepared by dissolving a premix (Ameroso, Solon, OH, USA) with 1 μg/ml ethidium bromide (EtBr) (Molecular Probes, Eugene, OR, USA) in deionized water (pH 8.3). EtBr was used as the intercalating dye in this case. Poly(ethylene oxide) (PEO) was obtained from Aldrich (Milwaukee, WI, USA). The sieving matrix was made by dissolving 1.5% 8 000 000 M_r PEO in 1× TBE buffer while stirring slowly. The solution was stirred for about 24 h and then left standing for 24 h until all bubbles vanished and a uniform and clear gel was formed.

2.2. Protocol for direct PCR from cheek cells

A sterile plastic toothpick (5 cm×2 mm) was used to collect cheek cells inside the donor's mouth. The sharp tip of the toothpick was rounded so that it would not hurt the inner wall of the mouth. No drinking and eating was allowed for the donors 1 h before cell collection. In addition, water was used to rinse the mouth one or two times just before cell collection. Cheek cells were collected by twirling the toothpick on the inner cheek for 30 times.

Two different approaches to perform PCR directly from cheek cells were developed according to how the cells collected on the toothpick were handled. In the first approach, named one-tube direct PCR, the toothpick with collected cells was immersed in 12.5 μl of PCR cocktails solution contained in a 200-μl polypropylene tube. The solution was vortex-mixed for 20 s to disperse the cells. A 250-nl volume of the solution, followed by 80 nl of Tris–HCl buffer, was immediately aspirated into the capillary (11.5 cm×

75 μm I.D.) and placed in the microthermal cycler to start PCR. In the second approach, named two-tube direct PCR, the toothpick was immersed in 12.5 μl of solution containing 4 μl of formamide and 8.5 μl of water. The solution was vortex-mixed for 20 s. A 125-nl volume of the solution and 125 nl of PCR cocktail were aspirated into the reaction capillary, dispensed back (dispensed volume of 260 nl) into a polypropylene tube for 40 s to mix the PCR reagent and the DNA template, aspirated (aspiration volume 250 nl) back into the capillary followed by the aspiration of 80 nl of Tris–HCl buffer. For two-tube direct PCR, the cell-containing solution may be stored at 4°C for up to 1 week before use. It should be noted that after storage the cell-containing solution must be vortex mixed just before aspiration to disperse the cells precipitated at the bottom of the tube. Otherwise, the cells cannot be reliably introduced into the capillary. The final composition of the reagent mixture for one-tube direct PCR is the same as that for two-tube direct PCR. Single-locus direct PCR was performed with the following components: 50 m*M* Tris, pH 8.3, 540 μg/ml BSA, 3 m*M* $MgCl_2$, 200 μ*M* of each dNTP, 0.5 μ*M* of each primer and 0.12 units/μl Taq polymerase enzyme. Multiplex-direct PCR conditions were slightly modified by adding primers in the concentration range of 0.4–0.6 μ*M*. The temperature-cycling protocol for the microthermal cycler is set as follows: initial holding, 6 min at 80°C; denaturation, 10 s at 80°C; annealing, 1 min at 54°C; extension, 1 min at 60°C, 30 cycles; final holding, 5 min at 60°C to assure all the products are annealed to their double-stranded form. The complete cycling protocol takes ca. 1.5 h to complete.

2.3. Instrumentation

Fig. 1 shows the basic schematic of the integrated instrument. The entire system consists of a microthermal cycler, CGE with gel loading system, and microfluidic control system.

2.3.1. Microthermal cycler

The microthermal cycler was similar to that used in previous work [10]. Modifications were made in such a way that a thin gas chamber (6 cm×3 cm×3 mm) was formed between two plastic frames. In addition, four pin holes were drilled through the center of four walls of the gas chamber with two of them used for fixing the position of a thermocouple and the others for guiding the reaction capillary (bare fused-silica, A: 11.5 cm×75 μm I.D.) (Polymicro Technologies, Phoenix, AZ, USA) through the gas chamber. After these modifications, installation of the reaction capillary became much easier and more reproducible, and the system reliability was greatly improved.

2.3.2. CGE and gel loading system

A bare fused-silica capillary (65 cm×75 μm I.D.) was used for DNA separation. The separation capillary was connected to one port of a polyether ether ketone (PEEK) micro-cross (O) with extremely low dead volume (36 nl) (Upchurch Scientific, Oak Harbor, WA, USA). The fused-silica capillary (C: 20 cm×100 μm I.D.) for gel loading was connected to the opposite port of the micro-cross. The syringe pump (P_3) (KD Scientific, Wood Dale, IL, USA) equipped with a 3-ml plastic syringe (Becton Dickinson & Co., Franklin Lakes, NJ, USA) was used to feed PEO gel via the capillary C and the micro-cross into the separation capillary B. LIF detection was used to monitor the separation of PCR products. The 514-nm line from an argon ion laser was focused onto the capillary detection window using an uncoated plano-convex lens (L) with 12-mm focal length. Fluorescence was collected by a 10× microscope objective (MO) (Edmund Scientific, Barrington, NJ, USA), reflected by a mirror (*M*), and filtered by a 600-nm long-pass filter (Edmund Scientific) prior to photomultiplier detection. The electrophoretic separation in the CGE system was driven by a positive high-voltage power supply (Glassman High Voltage, Whitehorse Station, NJ, USA).

2.3.3. Microfluidic control system

The fluidic control system consists of two syringe pumps (Kloehn, Las Vegas, NV, USA), a switching valve (Valco Instruments, Houston, TX, USA), three freeze–thaw (F/T) valves and a micro-cross. The syringe pumps were programmable liquid delivery systems. The syringe pump (P_1) equipped with a 25-μl microsyringe and a two-way valve was used to accurately aspirate and dispense nanoliter-volume liquid solutions for PCR. The PEEK tubing (Up-

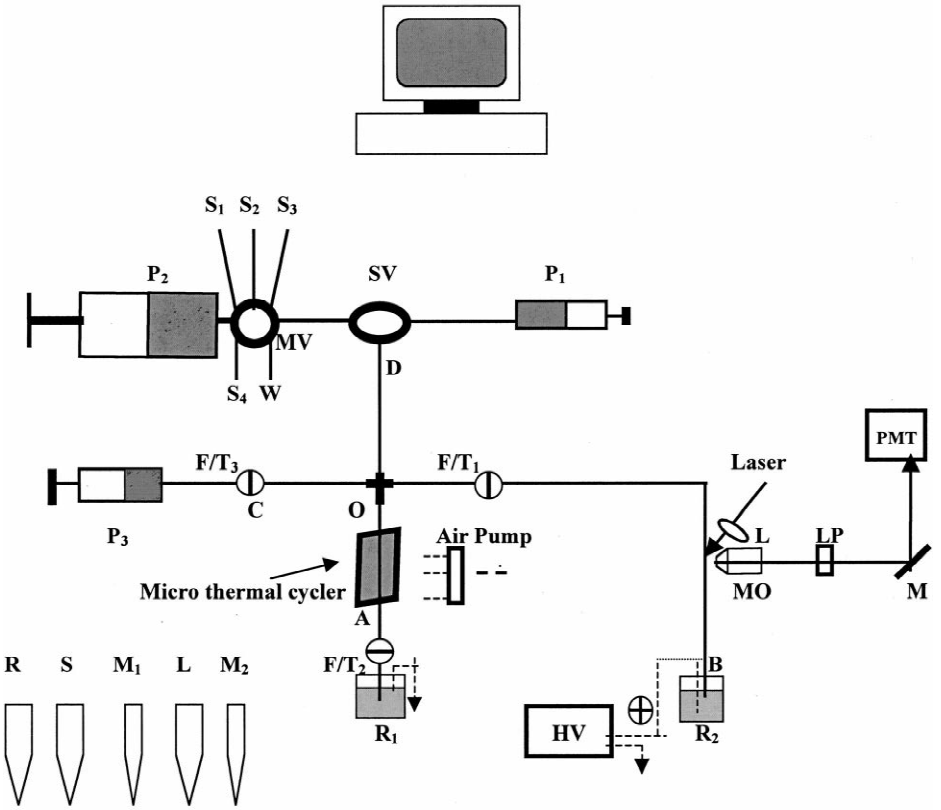

Fig. 1. Schematic of instrumental set-up. P_1, P_2, and P_3, syringe pumps; MV, six-position selection valve; S_1, deionized water; S_2, 0.2 *M* NaOH; S_3, Tris–HCl buffer; S_4, 1× TBE buffer; W, waste; SV, dual-position switching valve; F/T_1, F/T_2 and F/T_3, freeze–thaw valves; O, PEEK micro-cross; A, nanoreactor capillary; B, separation capillary; C, gel capillary; L, lens; MO, microscope objective; LP, 600-nm long pass filter; M, mirror; PMT, photomultiplier tube; HV, high-voltage power supply; R, S and L, tubes containing PCR reagents, DNA containing samples and sizing ladder; M_1, tube for mixing PCR reagents and DNA samples; M_2, tube for mixing PCR product and sizing ladder; R_1 and R_2, buffer reservoirs.

church Scientific) connected to the outlet of the two-way valve was glued to the port to prevent evaporation of liquid inside the valve. The syringe pump (P_2) with a 0.5-ml syringe and a six-way distribution valve was used to distribute large volume (milliliter levels) liquid solutions for conditioning and regenerating the reaction and separation capillaries. The center port of the six-way valve was connected to a 0.5-ml syringe. One port was connected to the switching valve (SV) via a fused-silica capillary (25 cm×150 μm I.D.), and the other five ports were connected via PTFE tubings to separate reagent bottles containing deionized water (S_1), 0.2 *M* NaOH (S_2), Tris–HCl buffer (S_3), 1× TBE buffer (S_4) and waste (W). The SV valve connected to the syringe pump P_1, P_2 and the micro-cross was deployed to direct the liquid from either P_1 or P_2 to the micro-cross via a fused-silica capillary (D: 20 cm×150 μm I.D.). The F/T valve system (Fig. 2) was built on the base of a thermal-electric cooler (TEC) (Melcor, Trenton, NJ, USA). The TEC utilizes the Peltier effect to move heat from one side of the TEC to the other, creating a cold side and a hot side. When the d.c. power is activated, heat is removed from the capillary at the cold junction to the hot junction by the semiconductor and dissipated by the water flowing through the brass tube. The liquid in the capillary is frozen, shutting off the F/T valve.

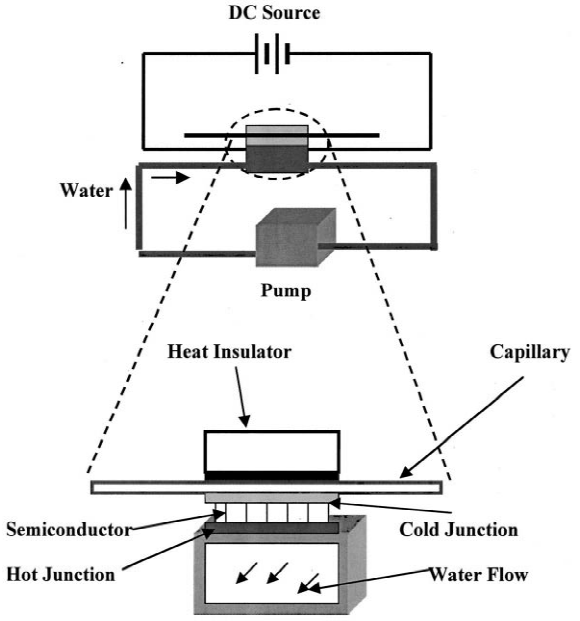

Fig. 2. Schematic diagram of freeze–thaw valve system.

When the power is off, heat is transmitted from the hot junction to the cold junction. The liquid in the capillary is thawed, opening the F/T valve.

2.4. Operation protocol

2.4.1. System conditioning and gel loading

(1) Initially, the SV valve was switched to the syringe pump P_2, the MV valve was directed to the port for water, the F/T valves were open and the syringe pump P_1 was filled with deionized water. (2) P_2 delivered 1 ml of deionized water to flush the whole system. (3) The F/T_1 valve was closed followed by the flushing of the reaction capillary (A) with 1 ml of 0.2 *M* NaOH. (4) The F/T_1 valve was opened followed by the dispensing of 1 ml of deionized water and 1 ml of Tris–HCl buffer from P_2. (5) The SV was switched to P_1 and F/T_2 was closed. (6) P_3 delivered 40 μl of PEO gel to fill the CGE capillary (C). (7) F/T_2 was opened, F/T_1 was closed, and the SV was directed to P_2. (8) P_2 delivered 1 ml of Tris–HCl buffer to flush the capillary A and to remove the gel in the capillary B. (9) The SV was switched to P_1, F/T_3 was closed, and the A inlet was immersed in the Tris–HCl buffer.

2.4.2. Reagents-sample loading and direct PCR

P_1 was deployed to load the PCR reagents, DNA sample and Tris–HCl buffer. For one-tube direct PCR, 250 nl of the reaction mixture with PCR cocktails and collected cheek cells in tube S was aspirated into capillary A followed by the aspiration of 80 nl of the Tris–HCl buffer in reservoir R_1. For two-tube direct PCR, 125 nl of PCR cocktail in tube R, and 125 nl of DNA samples in tube S was aspirated consecutively into capillary A, followed by their dispensing into the bottom of tube M_1. After a delay of 40 s for mixing by diffusion, the reaction mixture was aspirated into capillary A, followed by the aspiration of 80 nl of the Tris–HCl buffer. Then, F/T_2 valve was closed and the PCR process was started.

2.4.3. DNA ladder loading and sample injection

After PCR, F/T_2 was opened, and 70 nl of DNA ladder in tube L was aspirated into capillary A by P_1. Then, the PCR products and the DNA ladder in capillary A was dispensed (700 nl) to the bottom of tube M_2 for 40 s for mixing. A 600-nl volume of the mixture was aspirated into capillary A, and capillary A was immersed in Tris–HCl buffer in the buffer vial. The F/T_1 valve was opened and 9 kV was applied between reservoirs R_1 and R_2 for 40 s to inject the mixture into the CGE capillary (B).

2.4.4. Separation and detection of PCR product and DNA ladder

Immediately after injection of PCR product and DNA ladder, F/T_1 was closed, and the SV was switched to the P_2. A 100-μl volume of 1× TBE buffer was dispensed by P_2 to replace the Tris–HCl buffer in capillary A. Then, the F/T_1 valve was opened, the SV valve was switched back to P_2, and capillary A was immersed in the 1× TBE buffer. 12 kV was applied between vial R_1 and R_2 to start the size separation. The separated PCR products and DNA ladders were detected by LIF.

2.4.5. Cleaning and regeneration

(1) With all F/T valves open and the SV directed to P_2, 2 ml of deionized water was pumped by P_2 to

remove the gel in capillary B and to flush capillaries A and B. (2) With F/T_1 closed and the microthermal cycler set at 80°C, 1 ml of 0.2 *M* NaOH was dispensed to flush capillary A. (3) A 1-ml volume of 0.2 *M* NaOH was dispensed to flush capillaries A and B with the microthermal cycler set at 26°C. The subsequent steps (steps 4 to 9) are the same as those described in Section 2.4.1.

3. Results and discussion

3.1. Microthermal cycler

The conventional air thermal cycler has a large gas chamber (22 cm diameter×7 cm high) [22,23], and a fan is required to force air circulation for rapid heat transmission. In our previous work [10], thermal conductive silicone grease was applied onto the capillary surface and brass sheets were used for rapid and even heat transfer. The use of thermal grease makes installation of the reaction capillary difficult, and can potentially stain the inlet tip of the capillary. In the microthermal cycler, neither a fan nor thermal grease was needed, as heat transmission in the thin gas chamber (3 mm) is efficient enough to quickly heat or cool the capillary. Fig. 3 documents the reactor temperature as a function of time for direct PCR of genomic DNA. Cooling from the melting temperature (80°C) to the annealing temperature (54°C) takes 10 s, corresponding to a cooling rate of 2.5°C/s. Heating from the extension temperature (60°C) to denaturing temperature (80°C) takes 7 s, corresponding to a heating rate of 3°C/s. The cooling and heating rates are comparable to those of our previous system [10], but much faster than those

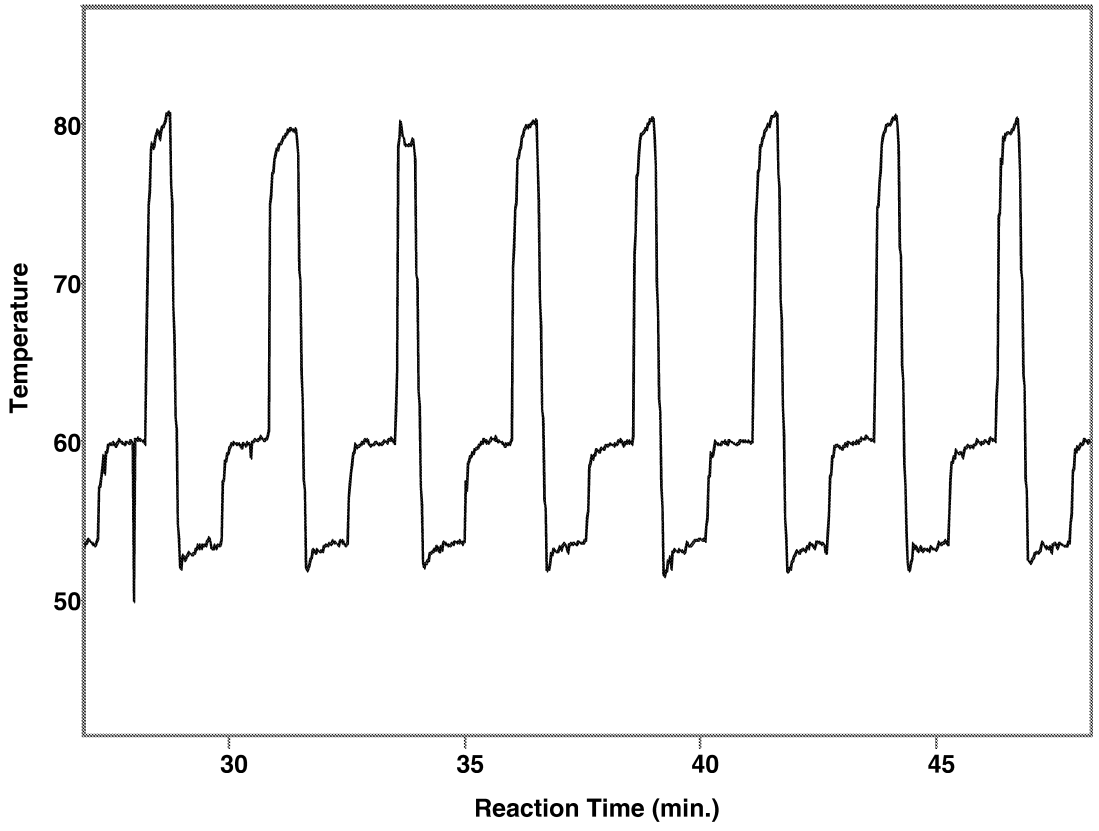

Fig. 3. Expanded view of the temperature profile of the microthermal cycler during the amplification of a 256-bp DNA fragment on the Y chromosome. The temperature profile was 80°C for 20 s, 53°C for 1 min, and 60°C for 1 min.

(1°C/s) of a conventional heating-block thermal cycler. In addition, the temperature profile showed good stability, with temperature fluctuations within ±0.2°C at the lower temperature range (40–75°C) and ±0.4°C at the higher temperature range (80–95°C).

3.2. Freeze–thaw valve system

In previous work [8,10], the liquid-nitrogen based F/T valve was closed by a flow of liquid nitrogen through a copper block where capillaries were placed, and opened by flowing hot air onto the copper block. Compared to mechanical valves, the F/T valve has the advantages of zero dead volume, no mechanical motion and good electrical isolation. However, manipulation of high-pressure liquid nitrogen raises some safety concerns, and the cost of operation is high. In this study, we developed a safe and inexpensive TEC-based F/T valve system (Fig. 2). To test its performance, the magnitude of the current through the capillary was used as the indicator of electrical flow and the size of the liquid droplet at the capillary outlet was used as the indicator of hydrodynamic flow. In addition, a thermocouple was used to monitor the temperature at the central point of the cold side of the TEC. We observed that the temperature decreased from 25 to −25°C within about 25 s and then stabilized around −25°C. The electrical flow was completely stopped when the temperature reached about −20°C. However, the hydrodynamic flow was stopped much later than the electrical flow. The response time for stopping electrical flow is thus ca. 20 s while that for hydrodynamic flow is ca. 2 min. The response time for opening the valve (both electrical and hydrodynamic) is very short, only 10 s due to the rapid heat transmission from the hot side to the cold side of the TEC.

3.3. Nanoliter direct PCR from cheek cells

Traditionally, DNA used for PCR-based analysis is extracted from white cells which in turn are

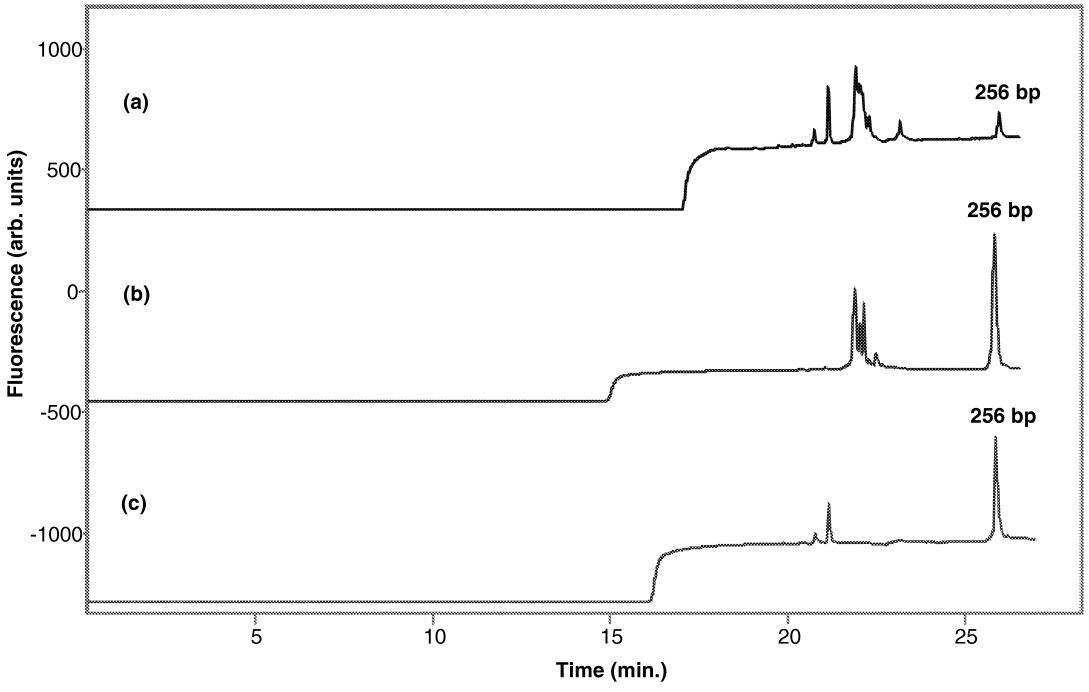

Fig. 4. Electropherograms of the amplification products from direct PCR using (a) fresh whole blood, (b) frozen and stored whole blood, and (c) fresh cheek cells. Separation matrix, 3% PVP; capillary, 70 cm (50 cm effective length)×75 μm I.D.; separation field strength, 150 V/cm.

fractionated from whole blood. Although this procedure can yield substantial quantities of DNA, blood-based methods have some drawbacks including the inconvenience of drawing blood and the risk of exposure to blood-borne pathogens such as HIV and hepatitis [24,25]. A variety of alternative sources of DNA have been used for genetic testing, including Guthrie spots, hair roots, urine samples, and cheek cells [26–29]. Cheek cell is perhaps the most extensively used non-blood-based DNA source. Oral saline rinses (mouthwash) and swab scrapings are two commonly used methods for collecting cheek cells. However, the mouthwash method requires an additional centrifugation step to spin down the cells, while the method based on swab scrapings needs a titration step to neutralize the concentrated NaOH, increasing the time and effort for DNA preparation. In this study, two simpler methods were developed for collecting cheek cells and performing direct PCR. For one-tube direct PCR, the cheek cells collected on the toothpick were dispersed in the reagent mixture for PCR without any intermediate treatment. The method is straightforward and is suitable for point-of-care diagnosis where immediate testing of the collected sample is required. For two-tube direct PCR, the collected cheek cells were dissolved in a water–formamide mixture. The sample collected in this way can be analyzed immediately or stored in a refrigerator for up to 1 week before use. This method also has the advantage of reducing the consumption of expensive reagents substantially as compared to the normal PCR carried out in polypropylene tube because reagents are introduced later and only at nl volumes. In both methods, thermal cell lysis to release DNA and to inactivate inhibitory substances was incorporated into the PCR protocol without a separate heating step. Finally, the reaction mixture is simpler than that for whole blood samples, since the more common Taq instead of Tth polymerase is utilized and binding proteins such as T4 gene 32 protein are not needed. As shown in Fig. 4, abundant amounts of products were obtained by direct PCR

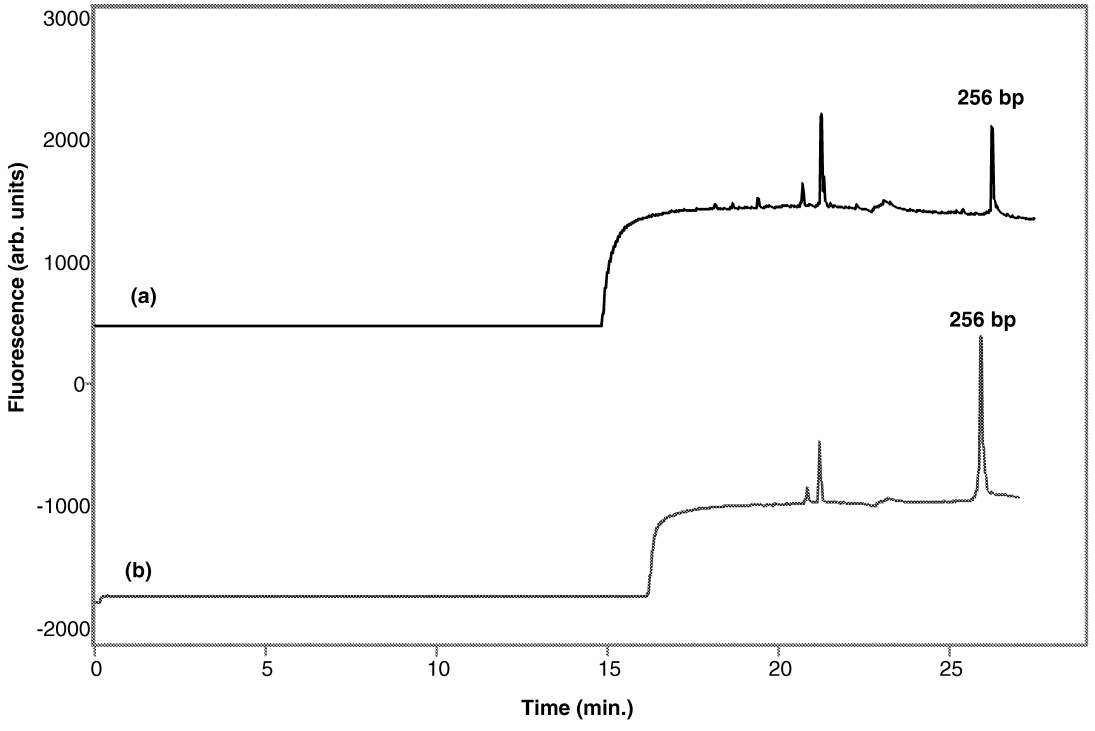

Fig. 5. Influence of the concentration of Taq polymerase on direct PCR in a capillary. Taq polymerase concentrations: (a) 0.04 units/μl and (b) 0.12 units/μl.

using DNA from frozen whole blood (stored at −20°C for 4 days) and from newly collected cheek cells. However, the product yield from fresh whole blood (ca. 1 h after blood drawing) is much lower. Moreover, the amplification specificity (extra peaks in the electropherogram) is worse than that from cheek cells. These results indicate that the activity of inhibitory substances in fresh blood may be higher.

In previous work on the direct PCR of DNA from whole blood [10], a long wide-bore capillary (40 cm×250 μm I.D.) was used as the reaction chamber with a reaction volume of 20 μl. In order to reduce the reaction volume to sub-microliter, a shorter capillary with smaller inner diameter was used here. It is well known that the capillary inner surface is an inhibitor of PCR, and the surface inhibitory effect increases when the I.D. decreases [30,31]. Surface inhibition can be reduced by adding high concentrations of BSA in the PCR mixture. With the addition of 540 μg/ml BSA in the reaction mixture, capillaries (24 cm long) with various I.D. (250, 150 and 75 μm) were tested off-line as the reactor for direct PCR. All reactions yielded ample products, and no significant difference in performance with respect to the capillary I.D. was observed. For direct PCR in 75 μm I.D. capillary, a higher concentration of Taq polymerase (0.12 unit/μl as compared to 0.05 unit/μl in normal PCR) was needed for adequate yield, as indicated in Fig. 5. This was different from direct PCR in polypropylene tubes where higher enzyme concentrations had no apparent influence on enhancing the product yield. The reason for this may be that the higher enzyme concentration compensated for the decrease in enzyme activity caused by the capillary inner surface.

The volume of a 24 cm×75 μm I.D. capillary is still over 1 μl. To further reduce the reaction volume, a shorter capillary (11.5 cm×75 μm I.D.) capillary was used. The reaction mixture was surrounded by Tris–HCl buffer at the two ends and was positioned in the middle of the reaction capillary. The plug length of the reaction mixture was ca. 5.5 cm with a corresponding volume of 250 nl. This is substantially smaller than the volume required by conventional PCR protocols. After thermal cycling, the reaction products were removed from the capil-

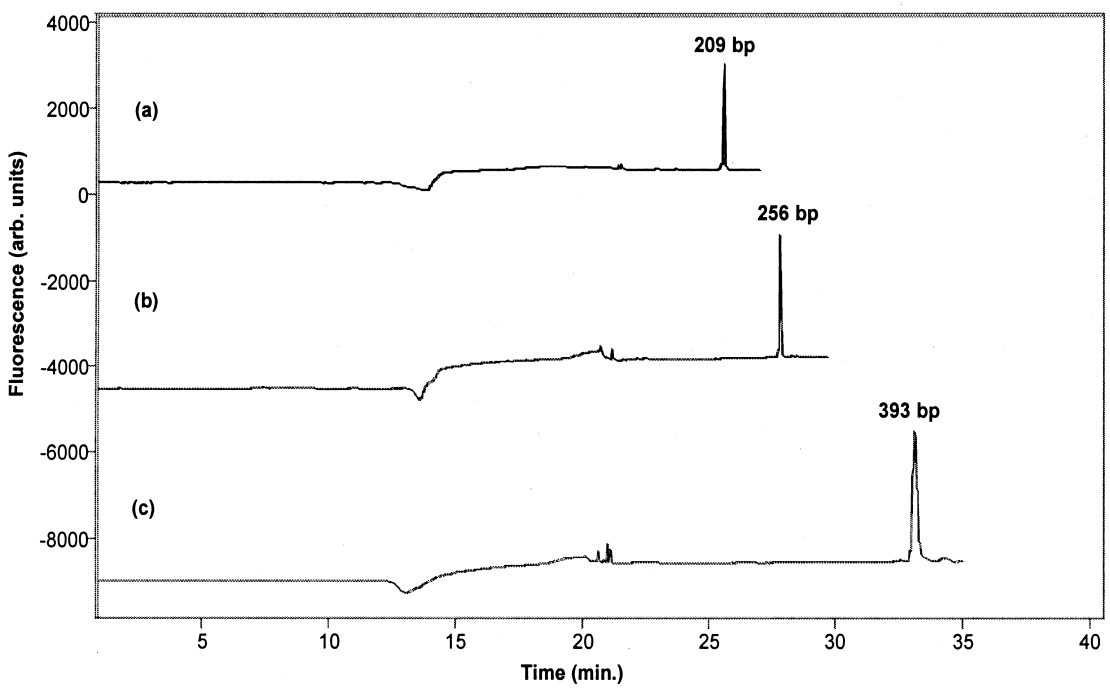

Fig. 6. Electropherograms of direct PCR products following on-line cell lysis and single-locus amplification of human genomic DNA on the Y chromosome. Targets: (a) 209 bp, (b) 256 bp, and (c) 393 bp.

lary, diluted by 3 μl of Tris–HCl buffer, and injected into the CGE capillary for analysis. As can be seen in Fig. 6b, the amplification products are readily detectable. In addition, no interference peaks were present, indicating good amplification specificity.

We performed single-locus direct PCR in capillary with a variety of primer pairs to determine the size of DNA fragments that could be amplified. The reaction efficiency and specificity of one-tube direct PCR are similar to those of two-tube direct PCR. For simplicity, only results for one-tube direct PCR are displayed (Fig. 6). As can be seen, amplification works equally well for long and short DNA fragments. The relative independence of the amplification efficiency on the DNA fragment size (200–400 bp) suggests that even longer DNA fragments should be amenable to amplification by direct PCR. Multiplexed direct PCR was also tested in capillary. For multiplexed amplification, there is competition among the different pairs of primers. There are differences in the optimum conditions for individual loci. Therefore, multiplexed PCR has much narrower tolerance limits than single-locus PCR. In this study, we found that the annealing temperature and concentration ratios of the primer sets were the key factors for obtaining strong signals for all three loci (Fig. 7).

For one-tube direct PCR of human genomic DNA, it should be noted that the volume of PCR reagents in the polypropylene tube is very important for collecting adequate concentrations of cheek cells. It was observed that one-tube direct PCR could not reproducibly yield adequate products if the volume of PCR reagents was over 25 μl. The reason for the irreproducibility may be because the cheek cells were not evenly distributed in the PCR reagents solution, and a minimum number could not be introduced into the narrow capillary if the cell concentration is not high enough. By reducing the volume to 12.5 μl, one-tube direct PCR could be performed in a reliable manner. This is also true of two-tube direct PCR of genomic DNA, where the

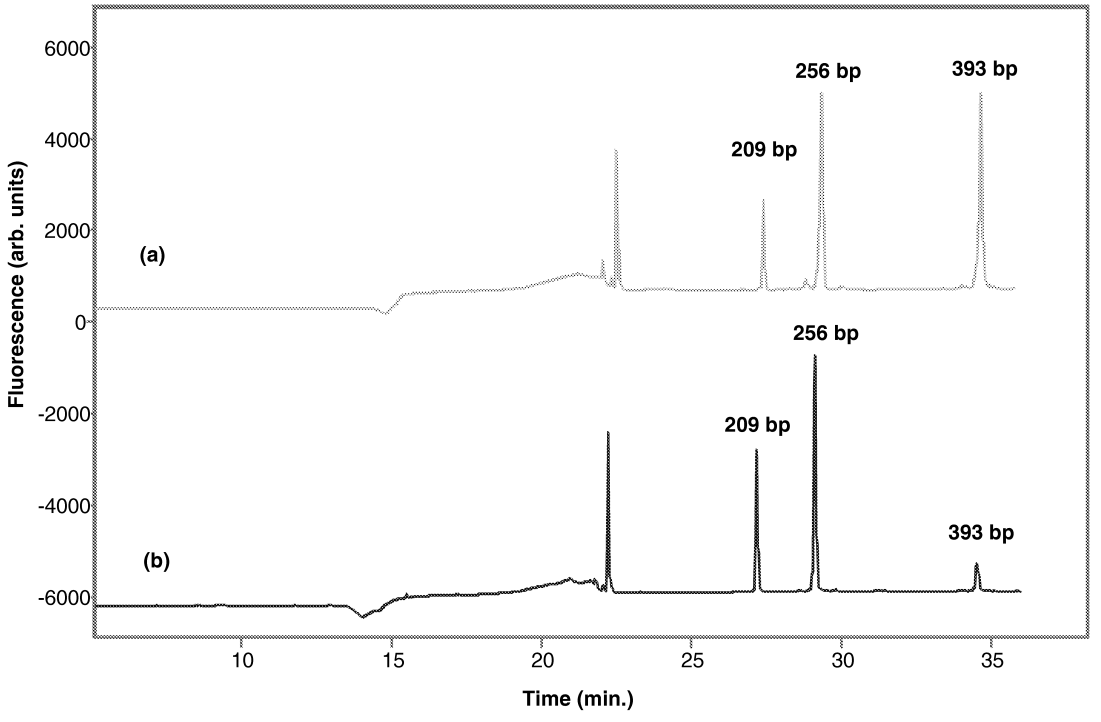

Fig. 7. Electropherograms of direct PCR products following on-line cell lysis and multiplexed amplification of human genomic DNA. Concentrations of primer sets for 209 bp and 256 bp are set at 0.5 μ*M*. Concentration of the primer set for 393 bp is (a) 0.6 μ*M* and (b) 0.4 μ*M*.

collected cheek cells are best dispersed in 12.5 μl of water–formamide mixture.

For two-tube direct PCR, mixing of small volumes of PCR reagents and cheek-cell containing solutions at the bottom of the narrow polypropylene tube is achieved by both dynamic agitation and static diffusion. Agitation predominates the mixing process during the aspiration and dispensing steps at speeds of 17 nl/s. After dispensing the PCR reagents and cheek-cell containing solutions, mixing at the bottom of the plastic tube is governed by the static diffusion. It was found that 40 s was sufficient for complete mixing of the PCR reagents and cheek-cell containing solution.

3.4. On-line injection, separation and sizing of PCR products

Optimized on-line injection is essential for consistent separation and sizing of PCR products in CGE. Migration times in CGE alone cannot be used for identifying DNA fragments, since the capillary surface can be very different among capillaries and even the same capillary can show variations from run to run. Therefore, DNA typing and PCR analysis usually needs appropriate size standards to help interpret the results. In previous works [7,10], co-injection of DNA standards was achieved by either simultaneous injection of PCR products and DNA ladder mixed in the same vial or sequential injection of DNA standards and PCR products from two different vials. Accurate movement of the injected solutions to the center of the micro-cross for sequential injection of DNA standards and PCR products became very difficult when the reaction volume was reduced to nanoliters. In the worst scenario, no PCR product was injected at all. This was because the very short plug of PCR products could easily pass the center of the micro-cross if the aspiration volume was slightly larger than expected or fail to reach the center of the micro-cross if the aspiration volume was smaller than expected. Although this problem

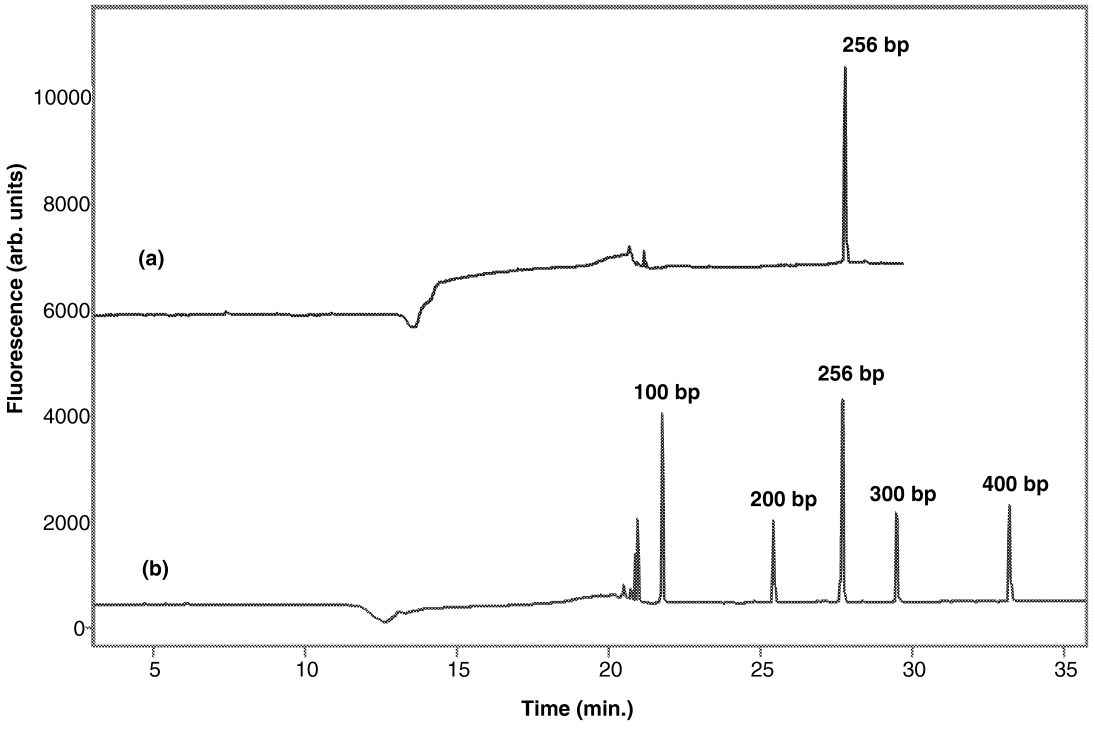

Fig. 8. Electropherograms of (a) direct PCR product amplified from human genomic DNA, and (b) direct PCR product mixed with the 100-bp sizing ladder.

could be partially resolved by increasing the injection time, a long injection time caused peak broadening and more serious variations in migration time. Here, reliable co-injection of small volumes of PCR products and DNA standards was performed as described in the Experimental section. Although the concentration of the PCR products was diluted by several folds before injection because of mixing, the new method guarantees the arrival of all DNA fragments at the center part of the micro-cross for injection. It was found that the variability of injecting the mixed DNA standards and off-line PCR products was less than 6.0%. Fig. 8 shows the electropherogram obtained from the on-line direct PCR, injection, separation and sizing of the PCR products from the 256-bp DNA fragment on the Y chromosome.

3.5. Regeneration of capillaries for PCR and CGE

The ability to reuse the capillaries for PCR and CGE is important for application of the capillary-based integrated system to routine DNA-based diagnosis. Carefully controlled washing is required to regenerate capillaries for CGE, and especially for PCR. Regeneration of the CGE capillary includes removing the old gel, cleaning the capillary, and restoring the surface silanol groups to their original protonated state. Regeneration of the PCR capillary reactor includes removing the numerous species in the PCR mixture, especially those surface-adsorbed proteins which will inhibit or bind the polymerase. In addition, post-reaction washing is necessary to eliminate the DNA template and all products from the previous reaction. Even extremely small amounts of residual PCR products will be amplified effectively by PCR during the subsequent run to cause false positive results. As the conditions to regenerate the PCR capillary are different from that for the CGE capillary, washing solvents and their flow paths should be carefully selected and controlled. It was observed that washing the reaction capillary with 0.2

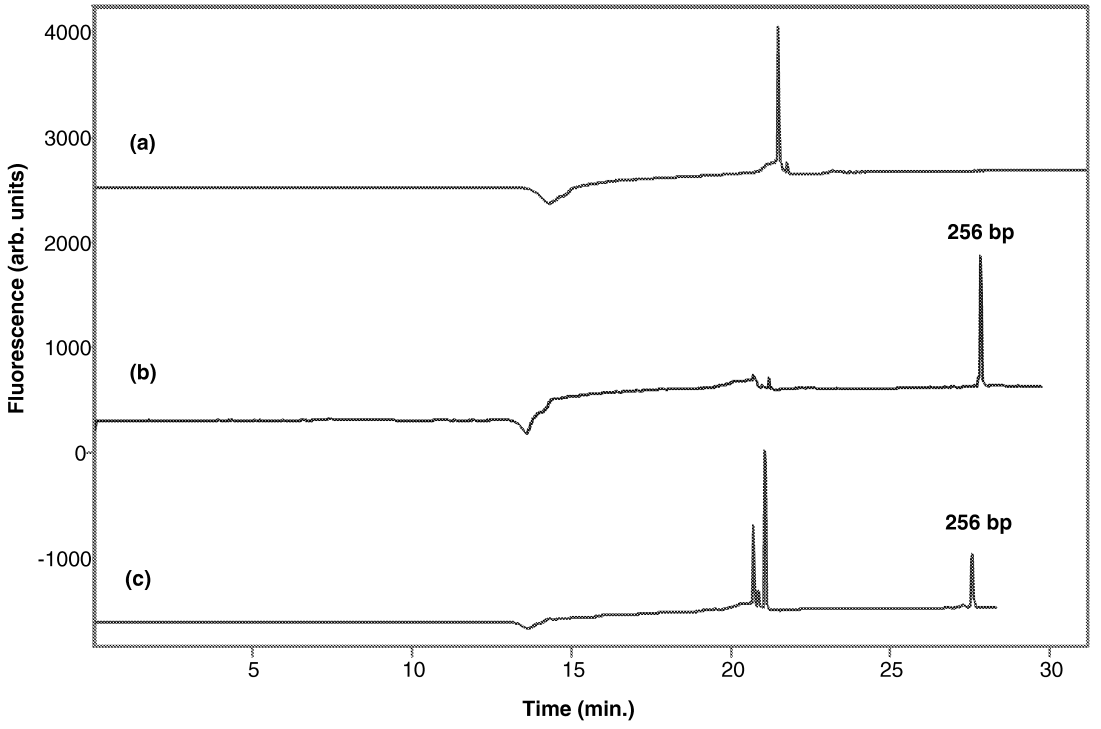

Fig. 9. Electropherograms of direct PCR products from (a) negative control (where no cheek cell is sampled), (b) DNA in cheek cells in a fresh capillary, and (c) DNA in cheek cells in a capillary which has been previously used for PCR for nine times.

M NaOH at 80°C and then at room temperature is necessary to completely remove the residual PCR products. However, flushing the CGE capillary with 0.2 *M* NaOH caused strong electroosmotic flow which could not be suppressed in the subsequent CGE separations. Hence, it is important that F/T_1 be closed when 0.2 *M* NaOH is flowing through the reaction capillary. The washing protocol described in the Experimental section was found to be highly satisfactory. This was demonstrated by performing negative-control PCR which gave no targeted DNA peak in the electropherogram (Fig. 9a). Additionally, 10 consecutive direct PCR were performed in this manner in one capillary. The product from the 10th reaction was still ample for analysis, although the reaction efficiency was somewhat lower (Fig. 9c). The amplification specificity was also preserved, as no interference peaks were present except that the primer–dimer was more effectively amplified as compared to that in the fresh capillary (Fig. 9b).

4. Conclusions

A capillary-based miniaturized, fully integrated and automated system for nanoliter PCR-based DNA analysis has been successfully demonstrated in the analysis of human genomic DNA directly from cheek cells. With reaction volume at 250 nl, the consumption of PCR reagents in two-tube direct PCR is only 125 nl. With the possible use of a capillary array in the future, the technique will provide an attractive approach for high-speed and low-cost DNA analysis.

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