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An integrated slidable direct polymerase chain reaction-capillary electrophoresis microdevice for rapid Y chromosome short tandem repeat analysis

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Abstract−We have developed an integrated slidable direct polymerase chain reaction-capillary electrophoresis (Direct PCR-CE) microdevice to analyze mini-Y chromosome short tandem repeat (mini-Y STR) typing without a DNA purification step. The Direct PCR has been recently commercialized as a simple DNA amplification technique, which does not require any sample preparation steps such as cell lysis, DNA extraction and purification for amplifying specific target genes. By implementing the advantages of Direct PCR on a microdevice, we can amplify target min-Y STR loci directly from human whole blood in a micro-reactor (2 μL) and the amplicons were successively separated by micro-capillary electrophoresis. The utilization of a slidable plate enables us to manually control the fluid without use of microvalves and microtubes, and the functional units of sample injection, Direct PCR, and CE analysis were sequentially and independently performed by switching the slidable plate to each unit. On the integrated slidable Direct PCR-CE microdevice, we could complete monoplex and multiplex mini-Y STR typing directly using human whole blood within 1 h.

Keywords: Microdevice, Direct Polymerase Chain Reaction, Capillary Electrophoresis, Mini-Y STR Typing

INTRODUCTION

Human identification testing with an aim of victim identification, missing person investigation, paternity testing, and sexual assault cases utilizes STR biomarkers due to high discrimination power [1-4]. Genetic analysis based on PCR in conjunction with CE is considered as a gold standard for STR typing. Contrary to the conventional STR typing process which requires large volume of reagents, prolonged reaction time, and bulky analytical instrumentations, genetic STR typing based on the lab-on-a-chip (LOC) technology holds strong advantages of high-throughput capability, cost and time effectiveness, high-speed reaction and analysis, full integration and portability [5-7].

Liu et al. [8] developed a fully integrated microdevice capable of performing sequence-specific DNA template purification, 250 nL PCR, post-PCR cleanup, and CE separation for rapid forensic STR analysis. This system can produce 9-plex STR profiles from 2.5 ng input standard DNA and obtain STR profiles from oral swabs in about 3 hours. Le Roux et al. [9] demonstrated a fully integrated sample-in-answer-out microchip for human identification based on STR analysis that includes a unique enzymatic liquid preparation for DNA extraction, a microliter non-contact PCR, and a high-resolution CE separation within a compact microchip footprint.

Although many researchers have established successful integration of the STR analysis on a chip, most of the reported microdevices relied on many microvalves and bulky external actuators for fluid control. In addition, they suffered from troublesome DNA extraction and purification steps, which made the entire operational process on a chip much complicated and cumbersome. Thus, chip operation should be simplified and the sample pretreatment process should be eliminated in order to construct a portable and reliable platform for STR analysis. To address these issues, we developed a novel integrated slidable Direct PCR-CE microdevice to perform mini-Y STR analysis without need of external pumping actuators and sample preparation steps. While the Direct PCR kit omits the DNA preparation steps including cell lysis, DNA extraction and purification to amplify specific target STR loci [10], the concept of a slidable plate allows fluidic control without microvalves. The amplicons produced in the Direct PCR zone were electrophoretically separated in the micro-CE (μCE) zone. The successive movement of the micro-PCR chamber from the sample injection zone to the Direct PCR zone to the CE zone could be manually manipulated by a slidable plate [11-13]. On the proposed integrated Direct PCR-CE microdevice, we could perform mini-Y STR analysis directly using human whole blood within 1 h.

MATERIALS AND METHODS

1. Design of the Integrated Slidable Direct PCR-CE Microdevice

The integrated slidable Direct PCR-CE microdevice consists of three layers, as shown in Fig. 1(a): from top to bottom, a slidable

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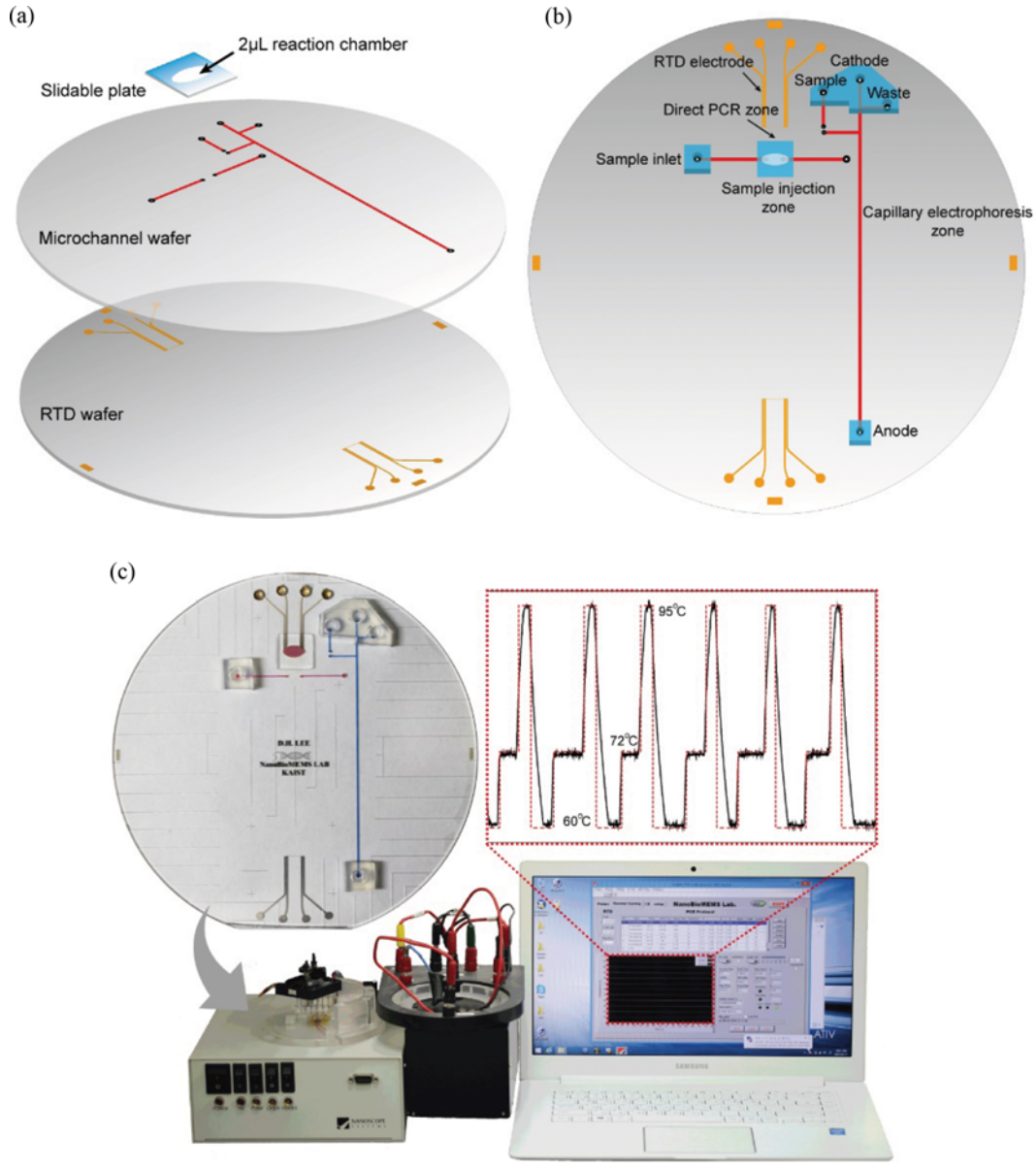

Fig. 1. (a) Exploded view of the Direct PCR-CE microdevice which consists of a slidable plate, a microchannel wafer, an RTD wafer from top to bottom. (b) Schematic image of the assembled Direct PCR-CE microdevice. The sample injection zone has a sample inlet, an injection channel, and a slidable plate. In the Direct PCR zone, an RTD electrode and a film heater are equipped. In the capillary electrophoresis zone, four electrodes (sample, waste, cathode, and anode) and a separation channel are patterned. (c) A digital image of the assembled integrated Direct PCR-CE microdevice and a portable genetic analyzer.

plate (10 mm×10 mm×0.7 mm), a 4-inch microchannel wafer and a 4-inch resistance temperature detector (RTD) wafer. On the bottom of the slidable plate, 2 μL of a concave shaped PCR chamber was fabricated, and the microchannels for sample loading and CE were patterned on the microchannel wafer with dimension of 140 μm width and 100 μm depth. The four-point RTD electrodes composed of Ti (2 nm thickness) and Pt (200 nm thickness) were deposited on the RTD water for real-time temperature measurement.

The assembled schematic image of the integrated slidable Direct PCR-CE microdevice is shown in Fig. 1(b). Three functional zones (a sample injection zone, a Direct PCR zone, and a capillary electrophoresis zone) are individually patterned on a single wafer. In the sample injection zone, the initially disconnected microchannels can be connected by placing the slidable plate in the middle, so that the reagent in the sample inlet reservoir can be loaded into the PCR chamber. In the Direct PCR zone, a film heater (2.2 cm× 0.6 cm, Kapton® Heater, MINCO, USA) is attached on the bottom of the RTD electrode for heating and the RTD electrode controls the temperature through proportional/integrator/differentiator (PID) module during the PCR thermal cycling [12,14]. The amplified DNA is electrophoretically separated in the micro-capillary electrophoresis zone, which consists of four PDMS reservoirs (sample, waste, cathode, and anode), a simple cross-injector and a 6.5-cm long CE separation channel [11,12]. The CE channels were also

originally disconnected, but, after the Direct PCR reaction, the slidable plate could be positioned in the middle to connect them. Fig. 1(c) shows a digital image of the integrated slidable Direct PCR-CE microdevice and a portable genetic analyzer (PCE-e, Nanoscope Systems Inc., Korea). In the integrated microdevice, the sample injection zone and the capillary electrophoresis zone are indicated as red and blue, respectively, and the slidable plate is placed on the Direct PCR zone (the PCR chamber is shown as red). The portable genetic analyzer can perform rapid and accurate thermal cycling for PCR and capillary electrophoresis with built-in high-voltage power suppliers, and amplicons signal detection with a portable fluorescence detector by an in-house LabVIEW program in the laptop computer.

2. Fabrication

The microfabrication process for the RTD wafer, the microchannel wafer and the slidable plate is similar to the previously reported literature [11,15]. A 4-inch borofloat glass wafer (PG&O, Santa Ana, CA, USA) coated with 200 nm of amorphous silicon was used to fabricate a microchannel wafer and the slidable plate. To promote adhesion of photoresist, the glass wafer was primed with hexamethyldisilazane (HMDS). After priming, positive photoresist (Microposit S1818 Positive Photoresist, Rohm and Haas Electronic Materials LLC, MA, USA) was spin-coated on the wafer surface and soft-baked at 120 °C for 90 s. The microchannel design on the film mask was transferred to the photoresist on the wafer by exposing UV and the exposed photoresist region was removed by a Microposit developer (1 : 1 with deionized water, Shipley). The revealed protective amorphous silicon region was anisotropically dry-etched by SF_6 reactive ion etching (RIE) and then, the microchannels were engraved by an isotropic wet etching using 49% hydrofluoric acid. The rest of the photoresist was washed away with acetone and the amorphous silicon layer was removed with KOH solution. Holes were drilled using a CNC milling machine with 1 mm diameter. To fabricate an RTD electrode wafer, 20 nm Ti and 200 nm Pt layer were deposited on the borofloat glass wafer and the photoresist pattern was formed by UV exposure and development. The residual Ti/Pt was etched in an aqua regia ($HCl : HNO_3$=3 : 1) solution at 90 °C and the remaining photoresist was stripped by acetone. The fabricated microchannel and the RTD electrode wafer were thermally bonded in a vacuum furnace at 668 °C for 5 h. The concave shaped 2 μL of a PCR chamber on the slidable plate was fabricated as described above on a borofloat glass and diced to a size of 10×10 mm. The PDMS reservoirs (sample, cathode, waste, anode, and sample inlet, blue color in Fig. 1(b)) were attached on the microdevice by oxygen-plasma treatment for 5 min. Finally, the surface of the microdevice and the slidable plate was hydrophobically treated with 30 μL of decyltrichlorosilane (DTS) for 1 h in a vacuum chamber to prevent sample loss during movement of the slidable plate.

3. Calibration of the Resistance Temperature Detector

Since the RTD electrode should precisely measure the real-time temperature during PCR thermal cycling, the RTD electrode should be calibrated prior to the PCR operation. The RTD electrode was calibrated as follows. The micro-patterned RTD electrode was submerged into a water bath and gradually the temperature was raised from 25 °C to 70 °C, while constant current (4 mA) was applied

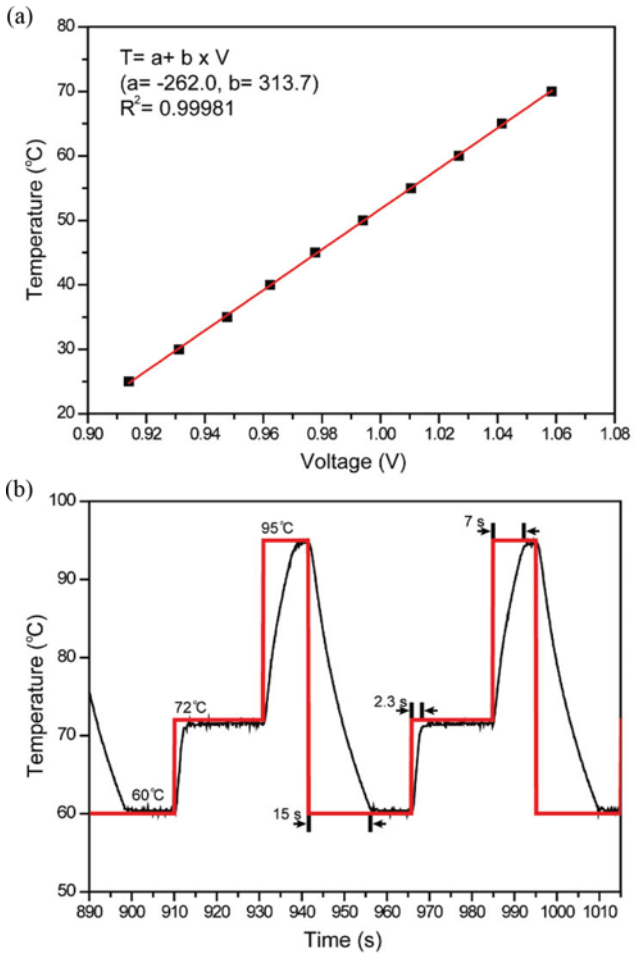

Fig. 2. (a) Calibration plot for RTD electrode. The temperature as a function of measured voltage is linear with an equation T= −262.0+313.7×V (R^2=0.999). (b) PCR thermal cycling profile. The red line is the set temperature and the black line is the measured temperature. Ramping rate was 5.2 °C/s for heating and 3.7 °C/s for cooling.

through the outside pair of Ti/Pt leads and the variation of voltage was measured by the inside pair of Ti/Pt electrodes. Fig. 2(a) shows the voltage change recorded every 5 °C as a function of the temperature. The temperature linearly increased with the electrical voltages, and their relationship can be expressed by

$$T=a+b\times V$$

where T is the temperature of the water bath and V is the measured voltage from the RTD electrode. From the linear regression plot, the constants a (−262.0) and b (313.7) were determined with high coefficient of determination (R^2=0.999). Using the above equation, the PCR thermal cycling profiles could be obtained as shown in Fig. 2(b). The temperature ramping rate was 5.2 °C/s for heating and 3.7 °C/s for cooling, thereby enabling rapid PCR [12].

4. Operation of the Integrated Slidable Direct PCR-CE Microdevice

The operation procedure of the microdevice is similar to our previous literature [11,12]. First, the slidable plate was aligned to bridge the initially disconnected microchannels in the sample injec-

tion zone. The slidable plate was surrounded with 5 μL of mineral oil (M8662, Sigma Aldrich) to prevent leakage and evaporation of the reagent during the Direct PCR reaction and the movement of the slidable plate. After 5 μL of a Direct PCR reagent was dropped into the sample inlet reservoir, and the reservoir was pressed down by finger to allow 2 μL of a Direct PCR reagent to be loaded into the PCR chamber as shown in Fig. 2(a). Then, the slidable plate was manually slid to the Direct PCR zone in which the PCR chamber was aligned at the center of the RTD electrode (Fig. 2(b)). Since the surface of the microdevice was hydrophobically treated, no loss of the reagents was observed. After attachment of the thin-film heater on the bottom, the Direct PCR reaction was carried out using an in-house LabVIEW program as follows: a lysis of cells and an initial activation at 95 °C for 5 min, 40 PCR cycles of 95 °C for 5 s, 60 °C for 10 s, 72 °C for 15 s, and a final extension step at 72 °C for 1 min. After the Direct PCR reaction, the CE microchannel was treated with 50% (v/v) dynamic coating in methanol (DEH-100, The GelCo., San Francisco. CA) for 3 min to minimize the electroosmotic flow, and was filled with 5% linear polyacrylamide (LPA) with 6 M urea as a sieving matrix (blue color, Fig. 2(c)). The temperature of the separation channel was kept at 70 °C by a silicon rubber heater during the CE separation. To apply high-voltage to the CE microchannel, 1×TTE buffer was added to the four PDMS reservoirs (sample, waste, cathode, and anode) and the slidable plate containing the Direct PCR products was moved to the CE zone in order to connect the disconnected part of the sample injection arm in the CE channel (Fig. 2(d)), and then a standard three-step CE operation was executed to analyze the mini-Y STR amplicons [11,12,16].

5. Preparation of a Direct PCR Cocktail

To accomplish the direct STR typing directly from human whole blood without DNA extraction and purification steps, we utilized a Human Tissue Direct PCR kit (Creative biogene, Inc.). The kit employs an engineered DNA polymerase enzyme with a DNA-binding domain that enhances the processivity of the polymerase and exhibits extremely high resistance to many PCR inhibitors found in human samples. Twenty μL of the Direct PCR cocktail included 10 μL of 2× an PCR buffer (includes dNTPs and $MgCl_2$), 5 μL of amelogenin and mini-Y STR primer sets (Table 1) [12,16], 1 μL of DNA polymerase (5 U μL^{-1}), 3.5 μL of deionized water and 0.5 μL of human whole blood. The primer concentration was optimized as 0.6 μM of amelogenin, DYS 393, and DYS 390 for 4-plex mini-

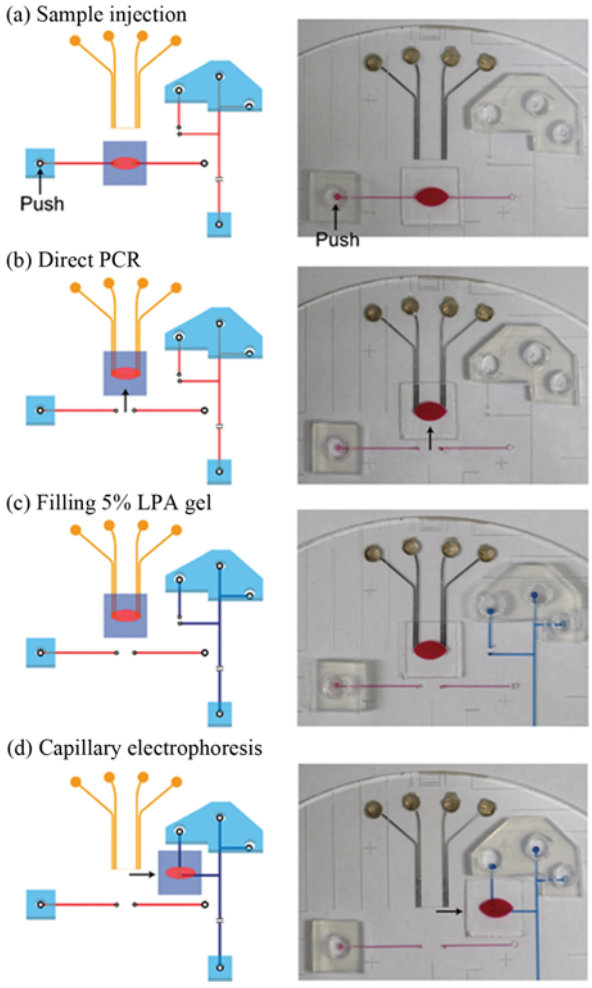

Fig. 3. The whole operation process of the integrated slidable Direct PCR-CE microdevice: (a) Sample loading into the slidable plate by pressing the PDMS reservoir with a finger (red color), (b) Direct PCR reaction, (c) filling 5% LPA gel in the CE zone as a sieving matrix (blue color), and (d) CE separation of the Y STR amplicons.

Table 1. Primer designs for mini-Y STR typing

Locus	Primer sequence (5′-3′)	Repeat motif	Allele range	Amplicon size
Amelogenin	FWD: FAM-CCCTGGGCTCTGTAAAGAA REV: ATCAGAGCTTAAACTGGGAAGCTG	[TCTA]	9-12	101-113
DYS 393	FWD: FAM-GTGGTCTTCTACTTGTGTCAATAC REV: AACTCAAGTCCAAAAAATGAGG	[AGAT]	12-15	120-132
DYS 389 I/II	FWD: FAM-CCAACTCTCATCTGTATTATCT REV: GTTATCCCTGAGTAGTAGAAGAAT	[TCTG]-[TCTA]	11-15/27-32	151-167/267-287
DYS 390	FWD: FAM-CTGCATTTTGGTACCCCATA REV: GCAATGTGTATACTCAGAAACAAGG	[TCTA]-[TCTG]	21-26	160-180
DYS 439	FWD: FAM-ACATAGGTGGAGACAGATAGATGAT REV: GCCTCAAGTGATCCACCCAAC	[AGAT]	10-14	184-200

Y STR typing. In case of 6-plex mini-Y STR typing, 0.3 μM of amelogenin, 0.8 μM of DYS389I/II, and 0.6 μM of DYS 390 and DYS 439 were used. The mini-Y STR primer sets were designed to produce smaller size of the resultant amplicons than those of the AmpFLSTR Y filter kit by shifting the primer binding sites to the amplified region as closely as possible [12]. Bovine serum albumin (BSA), which is typically used in the microchip based PCR to improve PCR efficiency and prevent nonspecific absorption of PCR components, was not added due to preexisting BSA in the human whole blood.

RESULTS AND DISCUSSION

1. Gender Identification Test on the Integrated Slidable Direct PCR-CE Microdevice

Since STR typing, especially in sexual assault cases, must be accompanied with gender identification test to confirm whether a sample originated from a male or a female source, we first conducted sex typing on the integrated slidable Direct PCR-CE microdevice. For this purpose, amelogenin gene marker was used, which revealed 106 bp of amplicon for X chromosome and 112 bp of amplicon for Y chromosome [17,18]. 0.5 μL of male or female whole blood were directly mixed with the Direct PCR cocktail and loaded into the PCR chamber as shown in Fig. 3(a).

Fig. 4 shows the electropherograms of amelogenin amplicons produced on the integrated slidable Direct PCR-CE microdevice. Male samples showed two distinct peaks with 6 bp difference, whereas female samples displayed one X chromosome peak. Therefore, we obtained two peaks (X and Y chromosome) discriminable from a male blood sample (Fig. 4(a)) and only one bigger peak (X and X chromosome) from a female blood sample (Fig. 4(b)). From these results, we verified that the amelogenin gene was well-amplified without a DNA purification steps as well as our integrated slidable Direct PCR-CE microdevice could be successfully applicable for STR typing.

2. Multiplex Mini-Y STR Typing on the Integrated Slidable Direct PCR-CE Microdevice

Encouraged by the successful gender identification test based on the integrated slidable Direct PCR-CE microdevice, we performed multiplex mini-Y STR typing on a chip. Sample injection, PCR amplification, and CE analysis were conducted in a consecutive order. 0.5 μL of male whole blood was directly used as a sam-

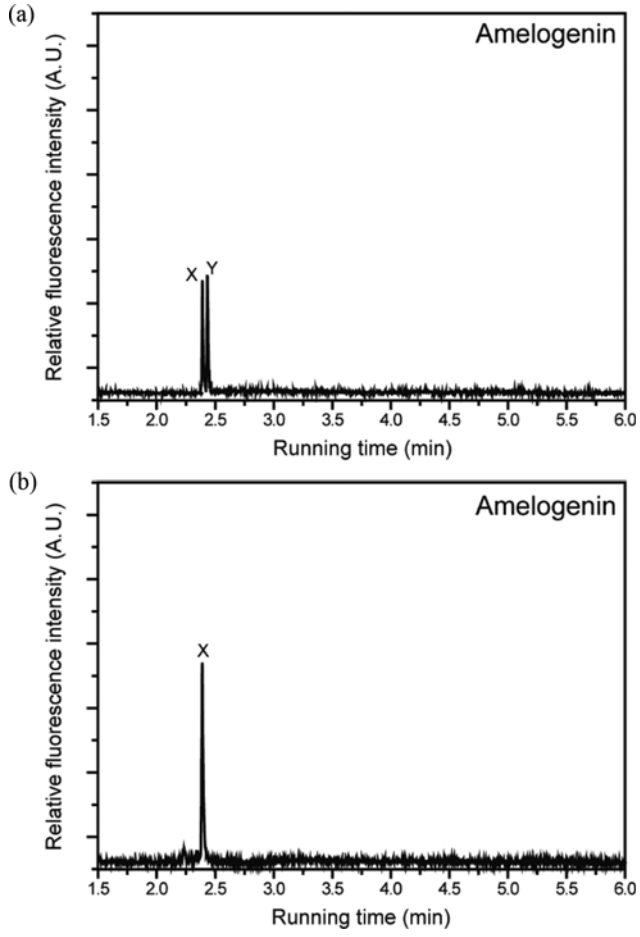

Fig. 4. Sex typing on the integrated slidable Direct PCR-CE microdevice. (a) A male whole blood sample shows discriminable two peaks, and (b) a female whole blood sample shows only one peak.

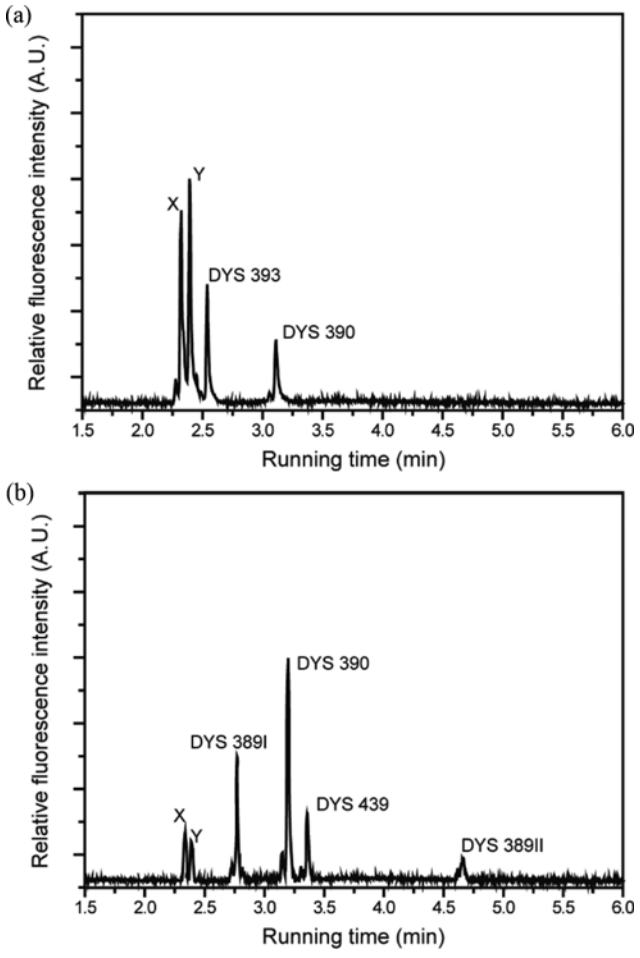

Fig. 5. Multiplex mini-Y STR typing on the integrated slidable Direct PCR-CE microdevice. (a) 4-Plex mini-Y STR typing and (b) 6-plex mini-Y STR typing.

ple without any pretreatment steps. 4-plex mini-Y STR typing targeting three loci of amelogenin, DYS 393, and DYS 390 was successfully performed as shown in Fig. 5(a). 6-plex Y STR typing targeting four loci of amelogenin, DYS 389I/II, DTS 390, and DYS 439 were also clearly analyzed without any non-specific peaks as shown in Fig. 5(b). Thus, these results demonstrated that our integrated slidable Direct PCR-CE microdevice enabled us to analyze multiplex mini-Y STR typing with high speed and high simplicity. We believe that further optimization of the chip design such as the channel length of CE can expand the multiplexing capabilities of STR typing on a chip.

CONCLUSIONS

We have successfully developed an integrated slidable Direct PCR-CE microdevice for rapid multiplex mini-Y STR typing. The Direct PCR reaction using human whole blood eliminates tedious DNA extraction and purification steps, and the use of the slidable plate enables fluid control without complicated microvalve systems. Sex typing and multiplex mini-Y STR typing could be finished on a chip within 1 h. It is expected that the proposed integrated slidable Direct PCR-CE microdevice would provide a novel forensic human identification analysis platform, which is particularly adequate for on-site STR typing.

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