INTEGRATED ALLELE-SPECIFIC POLYMERASE CHAIN REACTION-CAPILLARY ELECTROPHORESIS MICRODEVICE FOR SINGLE NUCLEOTIDE POLYMORPHISM GENOTYPING

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Abstract: In this study, we developed the polymerase chain reaction-capillary electrophoresis (PCR-CE) microdevice to perform the allele-specific (AS) amplification based on single nucleotide polymorphisms (SNPs) and its separation in one chip platform. Identification of the DNA finger print of HANWOO (Korean indigenous beef cattle) was carried out successfully on this device with an aim to distinguishing the HANWOO from the imported beef cattle. The on-site HANWOO identification can help the prompt prevention of zoonosis such as bovine spongiform encephalopathy (BSE) and efficient breed protection from imported beef cattle.

Keywords: Allele-specific polymerase chain reaction, Capillary Electrophoresis, SNP typing

INTRODUCTION

Single nucleotide polymorphisms (SNPs) are the most abundant genetic markers in the genome. As responsible for the diversity among individuals, they are intensively investigated for the potential biomedical applications such as therapeutic genomics and cancer diagnosis. In addition, SNPs are widely used to study genetic variations in animal as well as human [1]. Also in here, SNPs are used as efficient identification index of animals. Among the SNP genotyping methods, the allele-specific polymerase chain reaction (AS PCR) allows SNP to be detected in minimally equipped laboratories such as a thermal block and an electrophoretic stuff for DNA amplification and separation, respectively. Therefore, it can be easily integrated into the microfluidic system for the point-of-DNA analysis applications [2, 3].

Here, we fabricated PCR-capillary electrophoresis (CE) microsystem to identify the DNA finger print of HANWOO with allele-specific DNA amplification. For distinguishing the HANWOO from the imported beef cattle, HANWOO-specific five homozygous SNP sites are selected, and AS primers are designed with additional two primer sets for sex-typing and PCR control. With injection of designed AS primers and other PCR cocktails, the microdevice performs PCR in a 1-μL chamber and CE in a 6-cm-long separation channel under penumatic valve fluidic control. The successful development of this PCR-CE microdevice can provide a novel genetic analysis platform for SNP typing as well as HANWOO verification with high speed and sensitivity.

THEORY

AS PCR method relies on obtaining a PCR product specific to the SNP polymorphism using AS primers that have the 3'-end base complementary to the SNP site [2]. In addition, a couple of mismatched sequences adjacent to the 3'-end SNP site are designed for increasing stringency. Therefore, HANWOO-specific five homozygous SNP sites are selected, and AS primers are designed only for binding to heterozygous SNP sites which HANWOO doesn't have (Table 1). As a result, HANWOO should not show any AS peaks on CE while the imported beef cattle can reveal 3-5 AS peaks according to the varieties of beef cattle.

EXPERIMENTAL

Primer design for allele-specific PCR

Table 1 shows the primer design used in this study. MDH2 and SRY are malate dehydrogenase 2 and sex-determining gene for a positive PCR control and sex-typing, respectively. The allele-specific design of five primers (SNP 1, 8, 3, 6, and 7) for SNP typing is based on the previous report of Lee *et al* [4]. HANWOO cattle have homozygote in all five loci while imported beef cattle show heterozygote as well as homozygote. For this reason, primer sequences were tailor-made for specific heterozygote so that AS PCR is successfully performed only with imported beef cattle sample.

Microdevice design

The chip design and microfabrication process were based on the literature of Liu *et al*. [5]. A 1-μL PCR chamber and a 6-cm long CE microchannel are fabricated on the 4-inch borofloat glass wafer with a

Table 1: Primer design for allele-specific PCR

SNP or Gene	SNP No.	Genotype (HW[1])	Sequence	Amplicon (bp)
MDH2	-	-	F: ggggcctctggaggaattgg R: FAM-tgtgcctcctcaaagaatgg	100
SRY	-	-	F: cgaagacgaaagktggctct R: FAM-tgtgcctcctcaaagaatgg	122
ARS-BFGL-NGS-19963	1	C/**T**[2] (CC)	F: gccctgatcctgtggacactg R: FAM[3]-ccttttgcttataaaaatgatactcA[4]ta	185
BTB-01662048	8	**G**/T (TT)	F: FAM-cagatatatatgaaatacagatatatttCcg R: agagaataagatggagacaggaag	219
Hapmap33622-BTC-028272	3	C/**T** (CC)	F: tgtgtattcaatggacactgggt R: FAM-tttcctattatgaacaaaggttttttGgTa	250
Hapmap31109-BTA-141167	6	**A**/G (GG)	F: FAM-ccatggttactctttcctttccCCca R: tgggttgccttccacctactg	278
Hapmap46986-BTA-34282	7	C/**T** (CC)	F: gggtgggcacactttacaccaa R: FAM-tgactccctgaggactggataAGta	304

[1] HANWOO
[2] Allele specific primers are designed to be matched with the bold sequence (heterozygote).
[3] 5-Carboxyfluorescein dye
[4] Capital letters mean mismatched sequences at the 3' end of primers

four-point resistance temperature detector (RTD) and two poly(dimethylsiloxane) (PDMS) microvalves (Fig. 1). The bottom Ti/Pt RTD layer was thermal-bonded with the PCR-CE channel wafer, and the monolithic PDMS membrane and the manifold glass were assembled on the valve position.

For rapid thermal cycling during the PCR, an external flexible polyimide heater (2.2 cm × 0.6 cm, Kapton® Heater, MINCO, USA) and a fan were used for heating and cooling of a PCR chamber, respectively. A polyimide heater, a cooling fan, and Ti/Pt RTD electrodes were linked through DAQ boards (National Instruments, USA), and the automated temperature control and feedback was conducted by the LabVIEW software. A PDMS membrane was sandwiched between a channel wafer and a glass manifold to function as microvalves. Pneumatic channels for valve access were patterned in a glass manifold and linked with a solenoid pump for fluidic control. For CE separation, four high-voltage power suppliers were used, and each electrode was soaked in the sample, waste, cathode, and anode reservoirs. For denaturing the duplex PCR amplicons, a heater pad was placed on the CE separation channel, and the temperature was maintained at 70°C. Likewise, a thermal cycling system, a solenoid pump and high-voltage power suppliers were miniaturized to be packed in the 10×21×24 cm box (peripheral device unit, not shown here), and operated with a LabVIEW software.

PCR reagents and thermal cycling protocol

Beef cattle sample guaranteed by National Institute of Animal Science of Korea was purchased from a market. Genomic DNA from each sample was extracted by a solid phase extraction method using spin columns (QIAamp® DNA Mini Kit, QIAGEN, USA). The 10-μL PCR mixture contains 5 μL 2X multiplex PCR PreMix (Solgent, Korea), 3U H-Taq polymerase (Solgent, Korea), 30 ng extracted genomic DNA, and 1 μL premix of primers (Bioneer, Korea). The primer concentration for amplification of MDH2, SRY, and five allele-specific SNP sites varies for balance of peak intensities on electropherogram, ranging from 1 to 20 pmol/μL. The thermal cycling protocol on a microdevice was followed by an initial activation at 95°C for 15 min, 37 PCR cycles of 95°C for 20 s, 61°C for 40 s, 72°C for 60 s, and a final extension step for 3 min at 72°C.

Procedure for PCR and CE on a chip

Before loading the PCR cocktail, the sieving matrix, 5% (w/v) linear polyacrylamide with 6 M urea in 1X Tris TAPS EDTA (TTE) buffer, was injected from an anode reservoir with a syringe for filling a 6-cm CE separation channel. The PCR mixture was loaded into the sample reservoir, and then the PCR chamber was filled by suction at a vent reservoir. 1-μL PCR chamber was isolated by closing the active microvalves and a pssive valve derived from the LPA gel. For thermal cycling, the microdevice was fixed by the chip holder with 4 metal tips being contacted with RTD electrodes of a chip (Fig. 1(c)). A polyimide heater was attached on the position of a PCR chamber, and then the assembled microdevice was placed under the cooling fan of the peripheral device unit. After PCR, the CE separation was performed with the portable fluorescence detector (Nanoscope Systems, Korea). Each electrode (sample, waste, cathode, and anode) was soaked into the reservoir wells. An electric field of ~200 V/cm was applied between a sample and a waste reservoir for electrophoretic injection of the amplified PCR product. Following sample injection, a backbiasing step is followed to isolate a sample plug in

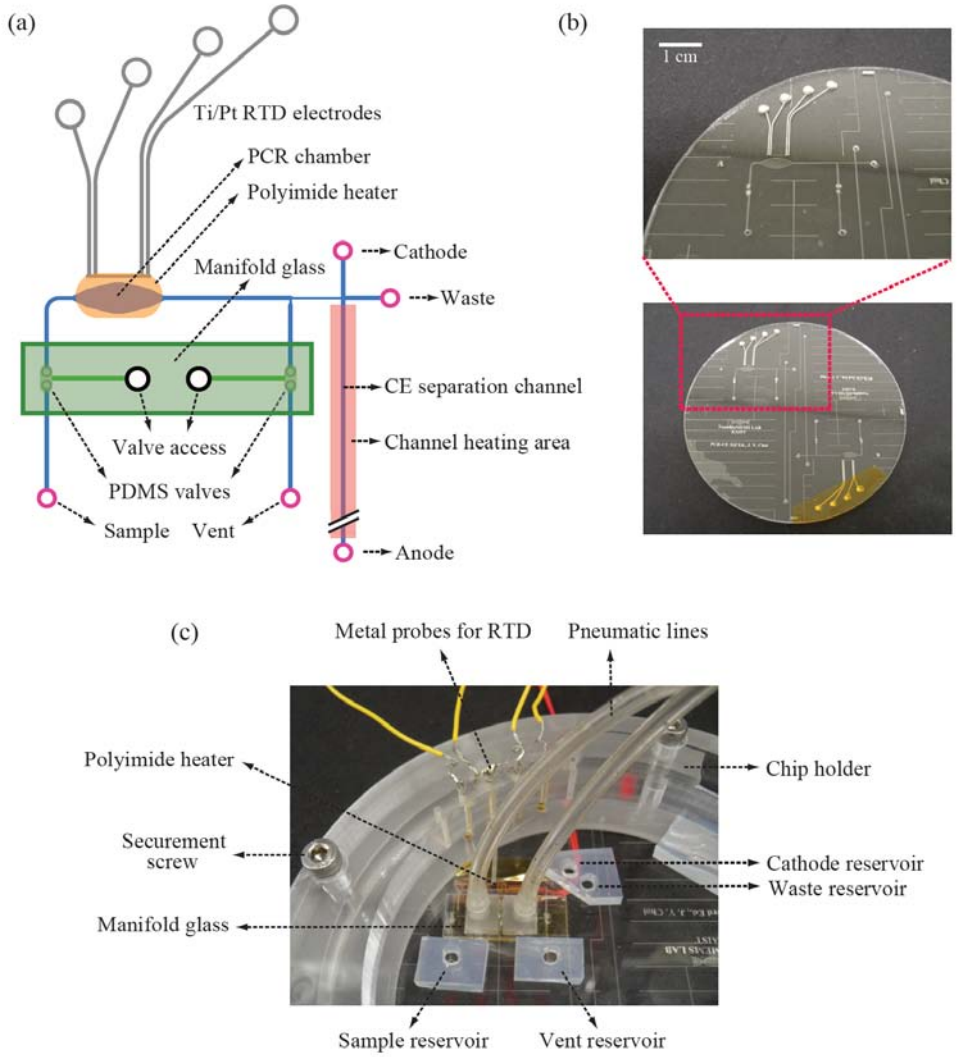

Fig. 1: (a) An integrated PCR-CE device design for PCR-CE. (b) A photogragh of a bonded wafer formed by a glass channel top wafer and a glass RTD bottom wafer. (c) A photograph of an assembled device with a bonded wafer, a polyimide heater, PDMS reservoirs, a manifold glass, a PDMS membrane (not shown), pneumatic lines, and a chip holder.

the intersection channel. Finally, an electric field of ~250 V/cm was applied between a cathode and an anode reservoir for separation. At the end of a separation channel, the fluorescence signal of the amplified products was detected on the portable fluorescence detector.

RESULTS AND DISCUSSION

Fig. 2 shows the thermocycling profile of the PCR-CE microdevice, meaning that red and black lines are set and measured values, respectively. Measured ramping rates for heating and cooling of a PCR chamber were 4.5°C/s (72°C to 95°C) and 2.5°C/s (95°C to 61°C). As shown, the miniaturized thermal cycling system works well according to the set value.

Fig. 3 presents the electropherogram of AS PCR product of beef cattle samples. Firstly, AS PCR was carried out on a conventional thermal block, and then products were separated using a CE on a micro CE chip which is exactly same microdevice with the CE functional unit of a PCR-CE chip (Fig. 1). As shown on a Fig. 3(a), the male HANWOO reveals two peaks, MDH2 and SRY meaning the PCR control and sex-typing of male. As expected, the HANWOO samples have no additional peaks induced by SNP typing, implying the sequence is C, T, C, G, and C on SNP 1, 8, 3, 6, and 7 sites, respectively. In the case of beef cattle, the product of AS PCR could be confirmed. In Fig. 3(b), there are all five products of AS PCR, meaning the sequence is T, G, T, A and T on same loci. In addition, the imported beef cattle showed no SRY

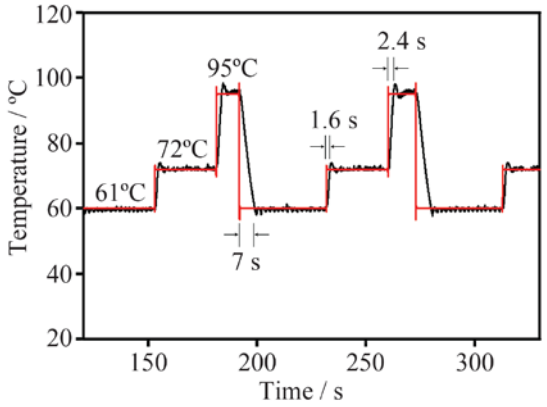

Fig. 2: Thermocycling profile of the PCR-CE micro-device

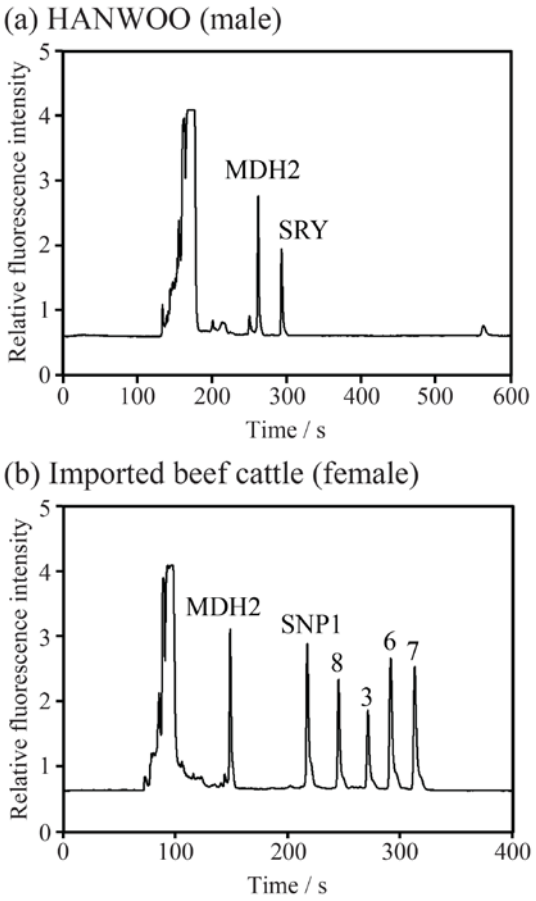

Fig. 3: Electropherogram of the beef cattle samples using a micro CE chip

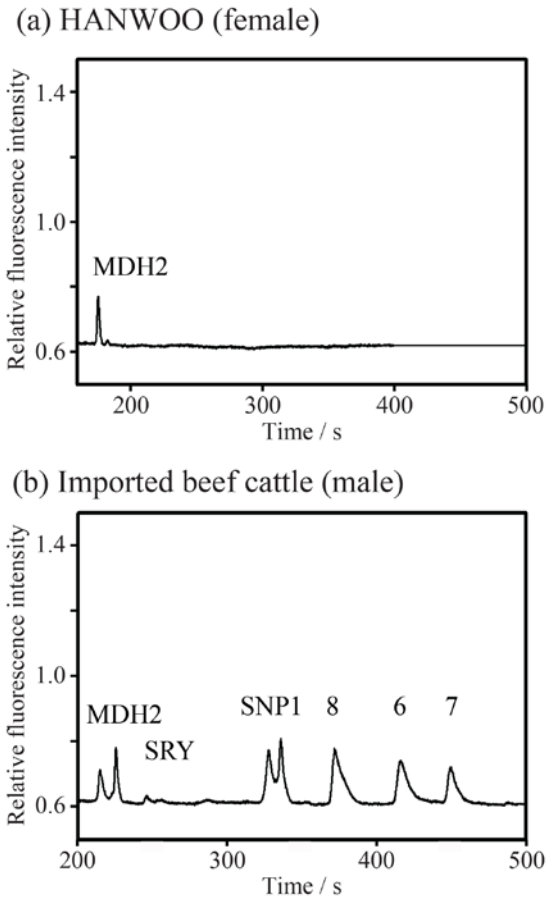

Fig. 4: Electropherogram of the beef cattle samples using a PCR-CE microdevice

peak, meaning the female sample. As shown in Fig. 3, successful SNP typing using AS PCR could be carried out.

Finally, the SNP typing using the integrated PCR-CE microdevice was performed. As shown in Fig 4(a), HANWOO male sample had only one peak, meaning the control while imported beef cattle produced four additional SNP products, meaning the sequence is T, G, C, A, and T on SNP 1, 8, 3, 6, and 7. Split peak of SNP1 proposes the further optimization of this system should be needed. But, a successful SNP typing using an integrated PCR-CE microdevice also could be performed within 105 min, showing the fast and efficient amplification and separation of target DNA.

CONCLUSION

We developed the polymerase chain reaction-capillary electrophoresis (PCR-CE) microdevice to perform the allele-specific SNP typing in one chip platform. With this microdevice, successful HAN-WOO verification using AS PCR could be performed. Additionally, these integrated Micro-systems can be applied for various biomedical research fields including point-of-care (POC) testing.

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