Melting analysis on microbeads in rapid temperature-gradient inside microchannels for single nucleotide polymorphisms detection^{a)}

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A continuous-flow microchip with a temperature gradient in microchannels was utilized to demonstrate spatial melting analysis on microbeads for clinical Single Nucleotide Polymorphisms (SNPs) genotyping on animal genomic DNA. The chip had embedded heaters and thermometers, which created a rapid and yet stable temperature gradient between 60 °C and 85 °C in a short distance as the detection region. The microbeads, which served as mobile supports carrying the target DNA and fluorescent dye, were transported across the temperature gradient. As the surrounding temperature increased, the fluorescence signals of the microbeads decayed with this relationship being acquired as the melting curve. Fast DNA denaturation, as a result of the improved heat transfer and thermal stability due to scaling, was also confirmed. Further, each individual microbead could potentially bear different sequences and pass through the detection region, one by one, for a series of melting analysis, with multiplex, high-throughput capability being possible. A prototype was tested with target DNA samples in different genotypes (i.e., wild and mutant types) with a SNP location from *Landrace* sows. The melting temperatures were obtained and compared to the ones using a traditional tube-based approach. The results showed similar levels of SNP discrimination, validating our proposed technique for scanning homozygotes and heterozygotes to distinguish single base changes for disease research, drug development, medical diagnostics, agriculture, and animal production. © 2014 AIP Publishing LLC.

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INTRODUCTION

Single nucleotide polymorphisms (SNPs), which represent the single base pair variations in a DNA sequence, are perhaps one of the most common genetic variations. Depending on where a SNP occurs at the coding regions, the mutated sequence alters the amino acid sequence of a protein and influences the original function of the protein. SNP is important and its associated studies have been widely conducted in disease research, drug development, medical diagnostics, selective breeding, as well as in agriculture and animal production.^{1,2} Lately, a number of SNP genotyping methods have exploded,³ including primer extension,^{4,5} enzymatic ligation,^{6,7} sequencing,^{8,9} and enzymatic cleavage.^{10,11} Although they can provide accurate results, they mostly require enzymes, molecular beacon, or fluorescent dyes to label the probes, resulting in high reagent cost and complex procedures.

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Meanwhile, DNA melting analysis has drawn great attention as an effective SNP genotyping method because of its excellent detection accuracy without the needs of expensive reagents and complicated procedures.^{12–16} As two strands of DNA, or duplex, “melt” when the surrounding temperature rises, the DNA strands, depending on the sequences, have the unique bonding strength and denature at their own melting temperatures (T_m). This melting analysis can serve as a vital technique in today’s genotyping and mutation scanning.^{17,18} Besides, microfluidic technology, which emphasizes the miniaturization liquid-handling systems to reduce sample/reagent consumption, thereby making the automation of complicated procedures possible and speeding up analysis time, has tremendously progressed in biological analysis.^{19–21} Thus, miniaturizing melting analysis onto a microchip can reduce the reagent cost and make personalized medicine possible.

DNA melting analysis in microfluidics usually resorts to a solid or liquid phase on sample preparations. Solid phase analysis allows for parallel reactions within a single microfluidic chamber, but it requires the immobilization of DNA or probes.^{22–24} Liquid phase analysis, on the other hand, eliminates the need of surface modification inside the devices, but it is restricted for one single analysis.^{16,25} To address the limitation and to enhance the reaction kinetics, micrometer-sized beads, which functioned as mobile supports, can be utilized.²⁶ Due to their mobility, these microbeads permit better flow dynamics and mixing efficiency, while preserve a high surface-to-volume ratio on the beads within the system. Further, using microbeads to immobilize the target DNA for melting analysis allows rapid heat transfer for the DNA samples to quickly reach the equilibrium temperature.

In addition, melting analysis requires the surrounding temperatures changing from 40 °C to 90 °C to denature the target DNA. There are two approaches: either time-based or spatial-based. The former technique employs a “heat-bide-image” heating protocol, which typically includes a temperature ramp (heat), followed by a temperature plateau (bide) when the fluorescence is measured (image). Detectors collect the data for durations of the DNA melting process. Melting analysis obtained by using quantitative Polymerase Chain Reaction (qPCR) or High Resolution Melt (HRM) techniques are the examples of this category.^{27,28} The latter technique is achieved by making a spatial temperature gradient for the target DNA and collecting the fluorescent signals as the melting data.^{29–31} In the time-based melting analysis, the speed of the detection is normally limited by the heating component of the device. One single analysis can only proceed after the previous one has been completed. In contrast, the spatial-based technique permits faster detection. It also reduces the thermal capacity due to the exclusion of the complete system from heating.

Herein, a bead-based DNA melting analysis, conducted in a temperature gradient environment, was proposed. A temperature-gradient region from 60 °C to 85 °C was created inside the microchannel. A heater was built on chip to allow a rapid thermal gradient in a very short distance with minimal energy required. Thermometers were also built on chip for *in situ* temperature measurement close to the DNA samples. The on-chip heater and thermometers precisely regulated the temperature on this microfluidic chip through feedback control for melting analysis. Afterwards, the microbeads with target DNA immobilized on their surface were carried by the flow continuously through the temperature gradient region for melting curve analysis. Genomic DNA from *Landrace* sows, which contained a SNP, ataxia-telangiectasia-mutated (ATM) gene, was tested using this bead-based SNP detection scheme to demonstrate the feasibility and potential applications in selective breeding. The ATM gene in *Landrace* sows played vital roles in the total number of piglets born, the number of piglets born alive, and the average birth weight of piglets due to its differential expression between the morula and blastocyst stages.^{32,33} Our bead-based SNP detection showed great potential as an effective approach in selecting useful biomarkers and in improving the reproductive traits in sows for selective breeding applications.

MATERIALS AND METHOD

Melting analysis and bead-based strategies

To simplify the SNP detection procedures, a short sequence spanning the SNP position, known as the Dynamic Allele-Specific Hybridization (DASH) technique, was adapted in this study.¹³ DASH employed an oligonucleotide allele-specific probe, which was designed and

intended to be complementary to the target sequence—a single stranded DNA (ssDNA). The probe hybridized with the target, which interacted with a double strand-specific intercalating dye, SYBR Green I. The non-covalent bonding between SYBR Green I and the target DNA would not interfere with the activity of nucleases and DNA polymerases. When heated, the double strand DNA (dsDNA) of the target sequence and probe would gradually denature with the intercalating dye being released, thereby decaying the fluorescent signals. The dye-emitted fluorescence was proportional to the amount of the dsDNA when excited. The relationship between the temperature and fluorescence intensity is named melting curve. Therefore, the normal DNA sequence and the mutated sequence had a different melting temperature (T_m), which resulted from the instability of mismatched base pair to specific designed DNA probe for discriminating the SNP.

Fig. 1 depicted the scheme of our bead-based SNP detection, which utilized the microbeads as mobile carriers to tether target DNA molecules and to flow through a temperature-gradient region inside microchannels. Prior to entering the microchannels, streptavidin-coated silica microbeads with 20 μm in diameter (Cat. 141048-05, Corpuscular Inc., Cold Spring, NY, USA) immobilized the biotinylated target ssDNA. The target ssDNA was hybridized with allele-specific probes, and intercalated with SYBR Green I to form a target-probe duplex. The microbeads with the target DNA were flowing through the channel, while an on-chip temperature control system was utilized to ensure a stable temperature gradient along the flowing direction, or lengthwise direction, of the microchannels. The microbeads and the DNA duplex moved across the temperature-gradient field and experienced temperature variations from 60 $^{\circ}\text{C}$ to 85 $^{\circ}\text{C}$. Thus, the DNA denatured with the fluorescent signals changing accordingly. The corresponding melting curves could be then obtained.

Microchip design

The design of the microchip was schematically shown in Fig. 2(a). The chip has a microchannel, a heater, and two thermometers. The microchannel was made of PDMS and bonded onto an ITO glass slide shown in Fig. 2(b), while the heater and thermometers were made of the ITO thin film on the slide. ITO was employed because of its advanced features in excellent transparency for fluorescence detection and in strong adhesion to glass substrates. Thus, the thermometers and heater were embedded onto the microchannel chip. The thermometers had one near the entrance of the detection region in the microchannel, while the other close to the

FIG. 1. Working principle of our bead-based melting analysis in a continuous-flow configuration. The DNA duplex, conjugated onto microbeads, passes through a temperature gradient region inside a microchannel. Every location in the channel can be related to a temperature. As a result, the microbeads and the DNA duplex on the bead surface experience temperatures ranging from low to high. The DNA duplex denatures, the fluorescent signals on the beads change, and they are then recorded. The melting curve can be then obtained. Note: red curve: melting curve of perfect-match sample; blue curve: melting curve of one-mismatch sample. (RFU: relative fluorescence unit.)

FIG. 2. (a) Top view of the SNP detection chip. Two thermometers and one heater were embedded in the chip and located close to the microchannel. The inset showed the close-up view of the detection region where the heater and thermometers were utilized to create a stable temperature gradient for melting curve analysis. (b) The sectional view of the SNPs detection chip.

highest temperature of the detection region (or close to the heater). The distance of these two thermometers, which corresponded to the length of the detection region, was deliberately chosen as $1250\text{ }\mu\text{m}$, to ensure the microbeads are monitored during the melting process within the vision field of the fluorescence detection system.

The microchannel was 2 cm long in total and designed in serpentine geometry to ensure stable flow fields during the detection. The height of the microchannel was $30\text{ }\mu\text{m}$ to allow only one microbead was flowing through at a time. The width of the microchannel was $300\text{ }\mu\text{m}$ to control the flow velocity below $100\text{ }\mu\text{m/s}$ with a micro-syringe and a syringe pump (NE4000, New Era Pump Systems, Farmingdale, New York, USA) at the flow rate of $1\text{ }\mu\text{l/h}$. To accommodate the microchannel within a limited space, the microchannel was designed in a serpentine geometry.

The ITO heater was employed to establish a temperature-gradient field in the detection region. As a result, it was designed to provide a uniform heat source with respect to the cross-section of the microchannels. The temperature around the heater was the highest and gradually decreased outwards. By controlling the current passing through the heater, the temperature field could be adjusted.

The thermometers employed the temperature coefficient property of the material. As the resistance of the material varied at different temperatures, the relationship between the resistance ($R(T)$) at the temperature (T) could be expressed as

$$R(T) = R_{T_0}[1 + \alpha(T - T_0)], \quad (1)$$

where T_0 was the reference temperature, R_{T_0} was the resistance at the reference temperature T_0 , and α was the temperature coefficient of resistance for the material. The temperature coefficient of the ITO in this study was measured and calibrated.

Fabrication

Standard microfabrication techniques were used to fabricate the heater, thermometers, and microchannels. An ITO glass of $7\text{ }\Omega/\square$ (UNI-WARD Corporation, Taiwan) was patterned to define the heater and thermometers by using photolithography and wet etching.

The microchannels were made using a soft lithography technique. The master mold was made on a (100) p-type 4 in. wafer. Negative photoresist of SU-8 2025 (MicroChem, Newton, MA, USA) was spun-coated on the wafer and defined the microchannels. PDMS (Sylgard 184, Dow Corning, Corning, NY, USA) was the main ingredient for the structure of the microchannels. Before the PDMS mixture (A:B = 10:1) was poured on the master and cured at 150°C , the wafer was coated with a diluted release agent, MAC-838 (McLube, Inc., Aston, PA, USA). The PDMS was peeled then punched to define the inlet and outlet of microchannels. It was then aligned to the region of the embedded heater and thermometers, and bonded onto the ITO

FIG. 3. An image of the SNPs detection chip.

glass using the partially cured bonding method.³⁴ The microchip then could be obtained as shown in Fig. 3.

Temperature measurement and feedback control

As a stable temperature gradient would be beneficial during the melting analysis, the temperature variation across a certain period of time on our chip was examined. An infrared camera (TVS-500EX, NEC, Inc.) was used to capture the thermal images and to realize the temperature distribution. A DC voltage was applied to the heater. Approximately 5 min was allowed for the temperature in the system to become stable. It was first confirmed that the temperature gradient within the detection region of the microchannel was perpendicular to the heater and in the lengthwise direction. Moreover, the measurements from the infrared camera agreed to the measurement done by using a digital thermometer (DE-3003, DER EE Electrical Instrument, New Taipei City, Taiwan). Since the temperature on the ITO glass surface could be measured, the temperature on the surface of the microchannel (T_c) could be estimated and calibrated.

The resistance and the temperature of the ITO thermometers were then continuously measured by using a data acquisition card (USB-6210, National Instruments, Austin, TX, USA). The temperature on the surface of the microchannel could therefore be real-time monitored by using the ITO thermometers on the chip. A feedback control was utilized to control the ITO heater. Visual programming language (LabVIEW 8.6, National Instruments Corporation, Austin, TX, USA) was employed as a Graphical User Interface (GUI) program for the temperature control system. The program contained three parts for achieving precise temperature control ability: (1) a Proportional-Integral-Derivative (PID) controller program, (2) a data acquisition and noise filter program, and (3) a power modulation program.

Target sample preparation

The SNP discrimination point ATM-A protein gene (Basic Local Alignment Search Tool: AY587061.1)—which was proven as a possible bio-marker associated with reproductive performance in *Landrace* sows, was chosen to demonstrate the validity and potential of our SNP detection system. The genomic DNA was isolated from blood samples of three individual *Landrace* sows using a PuregeneTM DNA Purification Kit (Cat. No. 15992, Gentra System, Inc., MN, USA). Based on a porcine nucleotide database (GenBank: AY587061), the primer pairs were designed. The translation starting site of the ATM gene was present within the exon 3.³⁵ To amplify the 5'-flanking region (upstream promoter and exon 1 to intron 2 region) of the ATM gene sequence, a two-step polymerase chain reaction (PCR) including symmetric PCR and asymmetric PCR was adapted. The amplicons and the probe were as below: ATM-A (C type), 5'-biotin-GGC TAC GTC CGA GGG TAG CAG CAT GAT CCA AGC CGC AGG AGT ACC CGC AGT GAG AGA CGA GAC TCA GGT AAA A-3'; ATM-A (T type), 5'-biotin-GGC TAC GTC CGA GGG TAG CAG CAT GAT CTA AGC CGC AGG AGT ACC CGC

AGT GAG AGA CGA GAC TCA GGT AAA A-3'; the allele-specific probe, 5'-CCT GCG GCT TGG ATC ATG CTG-3'. Furthermore, the results of the PCR products were sequenced for confirmation using an automated sequencer (ABI PRISM 3730 DNA Analyzer, Applied Biosystems Inc., Foster City, CA, USA). The nucleotide sequences were aligned for the SNPs detection using the program Lasergene (DNASTar, Madison, WI, USA).

PCR preparation

The ATM-A sequence was amplified from the genomic DNA extracted from sows. The amount of the DNA samples was more than 50 ng confirmed by the optical density (O.D.) value to meet the template requirement in PCR process. The ratio of absorbance at 260 and 280 nm ($A_{260/280}$) was kept above 1.8. All of the DNA oligonucleotides were confirmed by conducting the Polyacrylamide gel electrophoresis (PAGE) purification process after synthesis.

Symmetric PCR was first conducted with a forward primer 5'-CTT ACC CAA TAC CAG CCG GGC TA-3' and a reverse primer 5'-TTT TAC CTG AGT CTC GTC TCT CA-3' at the equivalent concentration. Each sample contained 10× PC2 buffer in 5 μ l, 50 mM betaine solution in 10 μ l, dimethyl sulfoxide (DMSO) in 0.5 μ l, 10 mM deoxyribonucleotide (dNTP) in 1 μ l, 10 μ M Forward-Primer (23 bp) in 1 μ l, 10 μ M Reverse-Primer (23 bp) in 1 μ l, Pro Taq Plus (PRO TECH, Inc.) in 0.5 μ l (5 units/ μ l), Q water in 26 μ l, and the template of the genomic DNA in 5 μ l. The symmetric PCR process was conducted with a thermal cycler (2720 Thermal Cycler, Applied Biosystems Inc., Foster City, CA, USA). The PCR conditions were: an initial denaturation at 94 °C for 5 min followed by 30 cycles at 94 °C for 20 s, at 55 °C for 20 s, and at 72 °C for 20 s.

In asymmetric PCR reaction, only Forward-Biotin-Primer—the sequence of 5'-Biotin-GGC TAC GTC CGA GGG-3'—was added into the reaction agent to amplify single-stranded target DNA. Since the products of symmetric PCR amplicons (91 bp) were used as a template in asymmetric PCR with the remaining Forward-Primer in the symmetric PCR solution, the Forward-Biotin-Primer was designed to be shorter, from 23mer to 15mer, to avoid erroneous amplification.

The total volume of the asymmetric PCR reagent was 50 μ l for each sample, which had 10× PC2 buffer in 5 μ l, 50 mM betaine solution in 10 μ l, DMSO in 0.5 μ l, 10 mM dNTP in 1 μ l, 10 μ M Forward-Biotin-Primer (15 bp) in 1 μ l, Pro Taq Plus (Pro Tech, Inc.) in 0.5 μ l (5 units/ μ l), Q water in 27 μ l, and the template of the ATM-A gene (91 bp) in 5 μ l. The asymmetric PCR process was conducted with the thermal cycle. The PCR conditions were the same as the ones in the symmetric PCR. The only difference was the temperature in the hybridization step, which was a little lower than the temperature in symmetric PCR due to the shorter primer length (55 °C vs. 52 °C).

Biotinylated ssDNA targets in three genotypes with ATM-A were then obtained. The 91-bp dsDNA and 73-mer ssDNA amplicons were verified by electrophoresis and compared with the DNA markers (25/100 bp mixed DNA ladder, Bioneer), shown as Figs. 4(a) and 4(b), respectively.

Melting protocol

Streptavidin microbeads (250 microbeads/ μ l) in 1 μ l, 2× SYBR Green I in 10 μ l, and 10 μ M of the probes in 1 μ l were mixed with ssDNA amplicons in 10 μ l from the previous two-step PCR amplification as the detection mixture. Before the mixture was loaded into the microchannel with a syringe (Cat. No. 80014, Hamilton Company, Reno, NV, USA), 1× PCR buffer would be flushed through the channel to maintain the ion concentrations. Silicone oil (polydimethylsiloxane, DMS-T21, η = 100 cSt, Gelest, Inc.) was subsequently injected until it filled a half of the microchannel (but not passed through the detection area) to pressurize the liquid flow and restrain the bubble formation at a high temperature. The temperature feedback system was then activated to create the stable temperature gradient in the detection region. Once the temperature distribution became steady, or the temperature variations were less than

FIG. 4. Electrophoretic images of three genotypes of amplified DNA from (a) the primary symmetric PCR and (b) the asymmetric PCR. (M: DNA maker, 1: CC, 2: CT, 3: TT, and N: negative control.)

0.15 °C, the mixtures were pushed again from the inlet at the flow rate of 1 μ l/h. The microbeads were transported through the temperature gradient.

The whole microchip was placed under an inverted fluorescence microscope (Axio Observer.A1, Carl Zeiss MicroImaging GmbH, Göttingen, Germany). The filters for excitation and emission were 425–475 nm and 600–660 nm according to the spectra of SYBR Green I. A series of the images were captured by using a cooled charge-coupled device (CCD) camera (Retiga R-2000, QImaging Corp., Burnaby, BC, Canada). The fluorescence signals of the microbeads that were transported through the temperature gradient could be obtained for the melting analysis. Once the detection was completed, DI water was flown into the microchannel for cleaning before the next detection mixture was introduced.

Signal quantification

The relative fluorescence intensity of the target-probe duplex on 20 μ m diameter microbeads was quantified from the images using image-processing software, ImageJ (National Institute of Health, Bethesda, MA, USA). The fluorescent images were converted to grayscale to calculate the fluorescent intensity of the microbeads. The fluorescence intensity was normalized as

$$\text{Relative fluorescence intensity} = \frac{X_i - X_{\text{final}}}{X_{\text{initial}}}, \quad (2)$$

where X_i was the fluorescent intensity of a microbead, X_{final} was the final fluorescent intensity of a microbead, and X_{initial} was the initial fluorescent intensity of a microbead. The melting curve profiles were normalized starting at 100% and ending at 0% and smoothed by using Savitzky-Golay (S-G) polynomials.³⁶ The first derivatives of the melting curves were calculated to find the melting temperature (T_m) for each genotype.

Genotyping validation

To validate our SNP genotyping results, a qPCR (Rotor-Gene Q, Qiagen, Inc.) system was used as a traditional approach to acquire the melting curve for the ssDNA samples in different genotypes. The ssDNA amplicons in three genotypes of CC, CT, and TT from *Landrace* sow were obtained after the asymmetric PCR. In each detection, 10 μ l of the ssDNA amplicon, 1 μ l (10 μ M) of the probes, and 1 μ l of the microbeads (250 microbeads/ μ l) were added into 10 μ l of 2 $\times$ SYBR Green I to become a total volume of 22 μ l for the traditional, tube-based melting analysis.

Once the ssDNA samples were ready, the samples were heated up to 95 °C for 60 s to denature the unspecific bindings. The melting analysis was carried out by cooling the temperature down to 55 °C and keeping at this temperature for 60 s. The temperature was gradually increased to 95 °C at 0.1 °C/s heating rate with the fluorescent signal measured for the temperature range between 65 °C and 85 °C.

RESULTS AND DISCUSSIONS

Characteristics of the temperature-gradient microdevice

The temperature distribution on the surface of the ITO glass along the microchannel was measured by the infrared camera, as illustrated in Fig. 5(a). To further examine the temperature fields where the microbeads were moving in laminar flow with the streamlines parallel to the lengthwise direction (y-axis) of the microchannel, the temperature distributions at three different widthwise locations (x-axis) were compared: one was compared at the centerline of the microchannel, while the other two were compared close to the channel sidewalls. As shown in Fig. 5(b), all temperature distributions at these locations were almost identical with a temperature variation of 0.06 °C, which showed negligible temperature variation in widthwise.

While the above temperature analysis focused on the ITO glass surface, the temperature distribution across the 30 μm height of the microchannel in the z-axis direction was also examined. Considering the microchannel material of PDMS was homogeneous, the temperature distribution in the cross-section of the microchannel in Fig. 4(c) was linear when the system reached an equilibrium. As the temperature on the glass surfaces (T_1) was measured as 85 °C by ITO thermometer, the temperature on the surface of the top PDMS layer (T_2) was measured as 50 °C by using the digital thermometer. The heat transfer rate could be estimated when the

FIG. 5. Temperature distribution of the SNPs detection chip was analyzed: (a) the infrared image illustrates the temperature distribution on the glass surface by using the heater. (b) The temperature distribution at three different cross-sections, where one was at the center of the microchannel, while the other two were at the channel sidewalls. All three cross sections showed almost identical temperature distributions, with a variation of only 0.06 °C. (c) The sectional view of the SNPs detection chip. Five specific temperatures were marked at five locations.

thermal conductivity of PDMS was 0.15 W/m K. Therefore, the temperatures at the top, center, and bottom locations in the cross section of the microchannel (T_{top} , T_{center} , and T_{bottom}) were estimated. The thermal conductivities of PDMS, ITO glass, and water were 0.15, 0.8, and 0.58 W/m K. T_{top} , T_{center} , and T_{bottom} were found as 84.15 °C, 84.41 °C, and 84.66 °C, assuming one-dimension steady-state conduction. As a result, the thin PDMS layer (20 μm thick with the spin-coating process) to insulate the microfluidic channel and the ITO thermometers caused only 0.34 °C temperature difference (T_{bottom} and T_1). This temperature difference would decrease when the temperature on the ITO surface (T_1) decreased, or when the locations were away from the heater.

Besides, a thermal gradient from 60 °C to 85 °C was obtained within a distance of 1250 μm (1.25 mm), compared to the thermal gradient from 60 °C to 95 °C in 10 mm, or from 20 °C to 100 °C in 12 mm where external heating was utilized.^{30,37} The on-heat heater not only allowed a rapid thermal gradient in a very short distance with minimal energy required but also prevented the heat loss in the substrate and insufficient thermal contact. Integrated with a microfluidic channel, the on-chip heater and thermometers enable rapid, efficient, and yet stable thermal process for DNA denaturation or melting analysis. This local heating, or rapid thermal gradient, also prevented other unintended areas on the microchip from increase of temperature, as the control of heat spread on a chip had been a difficult task.

Melting analysis

To acquire a stable temperature gradient field in the microchannel on the chip, the temperature feedback system with a PID control algorithm was employed to monitor the thermometers and to control the heater. DI water was continuously flown in the microchannel for 5 min until the readouts of the ITO thermometers reached the steady state. The mixtures, including the streptavidin microbeads, SYBR Green I, probes, and 73-mer DNA amplicons were injected into the microchannel. Prior to conducting the SNP detection, the silicone oil was pumped into microchannel from the outlet port to increase the pressure in the microchannel in case the bubbles formed. According to the geometry of the microchannel, the length = 2 cm, width = 300 μm , and height = 30 μm , with the flow rate controlled at 1 $\mu\text{l/h}$, the average speed of the internal flow in the microchannel 30.8 $\mu\text{m/s}$, and the maximum speed of the microbead along the center-line of the microchannel was 50 $\mu\text{m/s}$.

Fig. 6 shows a series of the fluorescent images of the microbeads flowing through the detection region. The images confirmed the microbeads, carrying the target-probe duplex and emitting the fluorescent signals in the microchannel from low to high temperatures. When the microbeads with the DNA duplex gradually travelled through the temperature gradient from 60 °C to 85 °C, the DNA duplex denatured and the intercalated fluorescent dyes were released, so the fluorescent signals decayed. The results confirmed that the DNA duplex denatured in this rapid thermal gradient region of 1.25 mm. The better heat transfer due to scaling allowed fast

FIG. 6. Fluorescent images of the bead in the microchannel at different locations, or temperatures, were superimposed. The bead was flowing from 60 °C in the right side to 85 °C in the left side of the image. Each image represented the bead with ~ 1.2 °C temperature difference. Also, the superimposed dotted line outlined the microchannel. The fluorescence intensity of the microbead gradually degraded when the temperature increased.

thermal equilibrium between the DNA/microbeads and stable surrounding temperature, making this DNA denaturation and melting analysis able to be quickly completed in 25 s.

Moreover, a number of fluorescence images from the multiple microbeads were collected and image-processed in grayscale. The fluorescence intensity data of the microbeads were quantified. Along with the locations of the microbeads, which was related to the temperature, the relationship between the temperature and fluorescence intensity was acquired. The melting curves were therefore plotted. Each microbead presented its own fluorescence variation during this melting process on its DNA duplex, which were immobilized on the bead surfaces. These melting curves were normalized and illustrated in Fig. 7(b), compared to the melting curves in Fig. 7(a) obtained by using the commercial qPCR machine in tube-based melting analysis. As depicted in Fig. 7(a), successful genotyping was confirmed. The CC genotype sample had the melting temperature (T_m) of 78.8°C and TT genotype sample had the melting temperature of 75.3°C. Compared to the T_m of homozygous and heterozygous samples, the ΔT_m was 3.5°C.

Using our SNP detection scheme, different genotypes of the target samples were tested. As shown in Fig. 7(b), at least three microbeads were selected for melting analysis in each genotype. The profile of the perfect-match sample (CC) had a lower decreasing rate in the beginning (between 60 and 67°C) due to the larger binding forces between the probe and target ssDNA. The maximum slope change, or the minimum value of the first negative derivatives of the curve, was determined as the melting temperature (T_m). The CC genotype sample had the highest melting temperature of 75.1°C, while the TT genotype sample had the lowest melting temperature of 67.1°C. The heterozygous CT genotype sample showed two melting temperatures at 67.2°C and 75.3°C. The melting temperature of the CT genotype (67.2°C and 75.3°C) was slightly different from those of the CC (75.1°C) and TT (67.1°C) genotypes.

The reliability of our SNP detection scheme was also examined. Fig. 8 summarized our results from different genotypes. The average value of the melting temperature from three

FIG. 7. The melting curves and the negative first derivatives ($-dF/dT$) of the three types of the ATM-A polymorphism (CC, CT, and TT) were obtained by using (a) Rotor-Gene Q and (b) our SNPs detection system.

FIG. 8. Melting temperatures of the three genotypes measured by our SNPs detection system.

independent experiments was $75.0 \pm 0.0^\circ\text{C}$ for the CC allele variant, with the average value of the melting temperature $67.5 \pm 0.5^\circ\text{C}$ for the TT allele variant being, as well as $76.3 \pm 1.7^\circ\text{C}$ and $67.2 \pm 0.3^\circ\text{C}$ for the CT allele variant. The p value of the two peaks was 7.9×10^{-6} , proving that these three genotypes could be statistically distinguished. In addition, compared to the T_m of homozygous and heterozygous samples, the ΔT_m was 8.0°C —larger than the results obtained by using traditional tube-based qPCR approach.

The genotyping results obtained by using our microchip (bead-based microfluidic, spatial melting analysis) and Rotor-Gene Q system (tube-based, temporal melting analysis) were consistent. The ΔT_m of 8°C by our bead-based microfluidic device was about 2.5 times larger than the ΔT_m of 3.5°C by Rotor-Gene Q system. Larger ΔT_m would be beneficial to distinguish the perfectly match duplex and the mismatch duplex. One of the main reasons for this advantage was that a faster heating rate was used. Given the flow rate and the temperature gradient in our device, the heating rate was about 0.62°C/s , which was higher than 0.1°C/s used in the tube-based system. While the heating rate increased, T_m shifted to a higher temperature. The matched configuration shifted more than the mismatched one did, resulting in the increase of ΔT_m .³⁸ In addition, because the heating rate would be affected by the flow rate, the microbeads were injected after the liquid flow was introduced and the temperature field was stabilized. It was recommended to have the same flow rate on the tests for one SNP location to ensure similar flow dynamics and heat conduction.

A rapid and stable temperature field, created by the embedded temperature control units that included the on-chip heater, thermometers, and PID control algorithm, also contributed better thermal performance in our device. The temperatures around the DNA samples and microbeads were locally accessed, leading to better measurement accuracy. Meanwhile, the microbeads permitted better local thermal stability and improved heat transfer due to scaling, compared to the liquid bulk inside the tubes from traditional qPCR approach. In addition, these microbeads concentrated the fluorescent signals from DNA onto a small area to enhance the signal-to-noise ratio, as well as provided better mobility and higher surface areas to improve the efficiency of interactions between samples and reagents, leading to better detection sensitivity.

Apart from larger ΔT_m , other advantages using bead-based SNP system included less reagent consumption and shorter detection time. This bead-based system utilized as few as three microbeads (or even one microbead) for melting curve analysis. Further, since the temperature gradient field had been created before the detection was conducted, the detection time—the time required to denature the DNA sample from 60°C to 85°C , or the time required for the microbeads moving across this region, could be decreased by increasing the flow speed of the beads. In this proposed scheme, the detection with one microbead was completed in less

than 6 min (5 min for temperature stabilization and less than 1 min for melting analysis), compared with 30 min by using Rotor-Gene Q.

The use of microbeads in the continuous-flow temperature gradient can bring the advantage in multiplex capability, compared to complicated procedures required in this aspect in our previous work.³⁴ Our devices presented in this article will allow one type of DNA samples immobilized onto one type of microbeads upstream first. Various types of the microbeads can carry different DNA sequences—while passing through the temperature from 60 °C to 85 °C to conduct individual melting analysis on each microbead in serial, so multiplex, high-throughput capability is achieved. This capability opens up the great prospect of multiplex detection of far more targets than the ones currently possible with conventional technology. Finally, this mechanism can potentially be adapted to a microfluidic droplet platform, where individual droplets enclose distinct target and reagents, with the same temperature gradient along the channel.^{39–42} The droplet functions like the microbeads as a mobile object to carry the target DNA and fluorescent dye for the melting analysis. While the droplet platform can possibly simplify the DNA immobilization step, the microbeads will provide better signal-to-noise ratio for this continuous-flow melting analysis in temperature-gradient channels.

CONCLUSIONS

SNP detection using DNA melting analysis was successfully conducted on continuous-flow microbeads in a temperature gradient field on a microchip. With an embedded heater and thermometers, a rapid temperature gradient was obtained between 60 °C and 85 °C within a distance of 1.25 mm. The distribution and stability of the temperature gradient were measured and analyzed. Further, as the microbeads immobilized with the DNA sample and intercalated fluorescent dyes passed through, the fluorescent signals decayed, which confirmed fast DNA denaturation and melting analysis. Different genotypes related to the procreation of piglets were tested. The genotyping results were evaluated with the melting temperatures (T_m) of the homozygous and heterozygous samples. This bead-based temperature gradient approach showed the accurate SNP discrimination ability, consistent to the conventional qPCR approach. The melting temperatures for the homozygous and heterozygous samples (of a 73-mer fragment) differed by 8 °C, which was two-fold larger than the differences obtained in conventional qPCR technique. This improvement in the melting temperature difference resulted from better temperature control, faster heat transfer, and higher heating rate. Finally, not only minimal reagent amount and simple sample preparations were required but also this scheme addressed the need of high-throughput SNP detection by the ability to sequentially have melting analysis by passing individual microbead with different DNA sequences in serial for multiplex capability, showing its great potential with high accuracy, high throughput for genotyping, and mutation scanning.

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