BEAD-BASED MELTING ANALYSIS IN TEMPERATURE-GRAIDIENT MICROCHANNELS FOR SINGLE NUCLEOTIDE POLYMORPHISMS DETECTION
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ABSTRACT
A novel single nucleotide polymorphisms (SNPs) detection scheme was proposed by conducting melting analysis on microbeads. These beads were utilized to immobilize target DNA duplex, and flowing through a well-controlled temperature-gradient region in microchannels on a microfluidic chip. As the microbeads going through from low temperature regions to high temperature regions, the DNA duplex denatured and the intercalated fluorescent dyes were released, inducing the fluorescent signals to decay. The melting curves were then acquired for SNP genotyping. Different genotypes (i.e. wild and mutant types) with a SNP location of Ataxia Telangiectasia-Mutated (ATM) genes from Landrace sows were tested and successfully discriminated.

KEYWORDS: Single nucleotide Polymorphisms, Ataxia Telangiectasia-Mutated gene, silica beads, continuously-flowing microchannels

INTRODUCTION
DNA melting analysis is one of the most vital techniques in today’s genotyping and mutation scanning [1]. Two strands of DNA, or duplex, denature when the temperature goes from 40°C to 95°C. Depending on the sequences, these duplex have different bonding strengths, leading their unique melting temperatures. Therefore, the ability to collect the melting information would allow researchers to gain greater detail in DNA sequence information. For instance, single nucleotide polymorphisms (SNPs) can be significant bio-markers, linking nucleotide sequence variations to phenotypic changes and helping researchers understand human diseases on a molecular basis. The SNP genotyping by employing the melting analysis has drawn great attention since it does not require the complex and expensive modification procedures on enzymes or on fluorescent molecules. Its detection results can be quickly obtained in high accuracy, compared to other SNP detection methods [2].

Meanwhile, in microfluidics, DNA melting analysis usually resort to solid or liquid phase on sample preparations, in which either DNA immobilization on channel surfaces is required or only single analysis is allowed [3]. To address the limitations, a bead-based melting analysis in continuous flow configuration is proposed. Silica microbeads are utilized as a mobile support to immobilize target DNA molecules flowing through a well-controlled temperature-gradient region inside microchannels for melting analysis. Using microbeads in microfluidics can enhance the reaction kinetics and permit better flow dynamics and mixing efficiency while preserve a high surface-to-volume ratio.

To evaluate the feasibility, a genetic analysis platform based on our bead-based detection scheme is established and facilitated with the utilization of wild and exotic genetic materials in animal improvement for practical applications. Ataxia Telangiectasia-Mutated (ATM) genes from Landrace sows, which play important roles in total number of piglets born, number born alive and average birth weight due to its differential expression between the morula and blastocyst stages [4], are tested with our platform. While our detection scheme successfully discriminates different SNP genotypes, it also shows great promise as a simple and powerful genetic analysis method.

THEORY
Our SNP detection scheme utilized the microbeads as a mobile support to immobilize target DNA molecules and to flow through a temperature-gradient region inside microchannels for melting analysis, as depicted in Fig. 1. Biotin-labeled target single-stranded DNA (ssDNA) A was immobilized onto streptavidin-coated bead, and the target ssDNA A was hybridized with allele-specific probe and intercalated with SYBR Green I to form a target-probe duplex. When the microbeads with the DNA duplex gradually passing 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 fluorescence intensity could be obtained and quantified from the images of the microbeads throughout the process; the corresponding melting curves for the target ssDNA A could be acquired.

EXPERIMENTAL
The detection chip consisted of an Indium Tin Oxide (ITO) glass coated with a thin film of polydimethylsiloxane (PDMS) and bonded onto a PDMS slab with microchannels. Before bonding, the ITO glass was patterned to define microheaters and temperature sensors, as shown in Fig. 2(a). The temperature sensors were calibrated using...
micro-infrared (micro-IR) camera and thermocouples. The temperature distribution of the microchannel was depicted in Fig. 2(b). A PID control scheme was adapted and created a stable temperature gradient inside a microchannel, as shown in Fig. 2(c) to in-situ monitor the temperature variation during the experiment. Fig. 2(d) further confirmed stable temperature distributions throughout the SNP detection process.

![Fig. 1. Working principle of the melting analysis with a continuous-flow bead-based microfluidic device for SNP genotyping: (a) The DNA duplex sample was loaded into the temperature gradient region inside a microchannel. The fluorescent intensity was observed, and (b) melting analysis was conducted and obtained. (T_m1: melting temperature of perfect-match sample, T_m2: melting temperature of one-mismatch sample) (RFU: relative fluorescence unit.)](image1)

Biotinlabeled ssDNA targets for two common genotypes of Landrace sows with position of the interest, ATM-A, were obtained after the symmetric and asymmetric PCR processes. The 21-mer probe designed complementary with the target ssDNA specifically bounded with target DNA. The target-probe duplex DNA was mixed with SYBR Green I stain then immobilized on the streptavidin microbeads. The mixtures were injected into the SNP detection chip by syringe with 1μL/hr. The microbeads were observed by using an epi-fluorescence microscope (Axio Observer, Ziess, Germany). Their motions and the fluorescence images were recorded via a CMOS camera (ORCA-Flash4.0, Hamamatsu, Japan). The fluorescence intensity on the microbeads gradually decreased when the temperature increased from 60°C to 85°C in a 1250μm-long microchannel.

The fluorescence intensity of the duplex on the microbeads was then quantified using ImageJ (National Institute of Health, Bethesda, MA, USA). The images were converted to grayscale. The images were then normalized. The differential data of the melting curve was also processed for explicit obtain the T_m of each genotypes.

![Fig. 2. (a) The ITO patterned glass bonded and aligned with the PDMS microchannel (Width = 300μm, Height = 30μm). (b) The infrared image illustrates the temperature gradient, constructed by the heaters. (c) The overview of the continuous flow SNP detection system. (d) Steady state evaluation on temperature variation throughout the time duration for SNP detection.](image2)
RESULTS AND DISCUSSION

The SNP genotyping analysis of each sample was performed using our bead-based detection scheme and commercial tool of Rotor-Gene Q instrument (tube-based) by monitoring the fluorescence intensity of the target-probe duplex with the temperature ranging from 60 °C to 85 °C. The fluorescence intensity data was then quantified and plotted, as shown in Fig. 3. The profile of the TT type chained down faster than the one of the CC type due to weaker binding forces between the probe and target ssDNA. The maximum slope change of the melting curves was determined as the Tm. The Tm of the TT and CC types were 60.5°C and 67.5°C respectively by using our bead-based scheme, compared to 75.6°C (for the TT-type sample) to 78°C (for the CC-type sample) by using Rotor-Gene Q. Our platform using the bead-based detection gave similar levels of discrimination on the results and enable unambiguous allele scoring.

![Fig.3: Melting curves for the samples by (a) our SNP detection system and (b) Rotor-Gene Q System.](image)

In addition, although the genotyping results from both were consistent, larger ΔTm value of 7°C between CC and TT types were obtained in our bead-based systems. Because of the faster heating rate could cause the larger ΔTm [5], the bead-based microfluidic system resulted in the larger ΔTm value, which can be beneficial to distinguish the perfect match duplex and the mismatch duplex, compared to ΔTm of 2.4°C from Rotor-Gene Q. Based on the flow speed of the microbeads and the temperature distribution inside the microchannel, our system was estimated to have more than 0.5°C/second heating rate during the SNP detection procedures, compared to 0.1°C/second from the Rotor-Gene Q system. Further studies are required to decipher the mechanisms of this larger ΔTm.

CONCLUSION

In conclusion, our approach employs microbeads for melting analysis in microfluidic devices and successfully distinguishes the wild and mutant genotypes of the samples. The utilization of microbeads not only provides a relatively high surface-to-volume ratio, but also enhances reaction kinetics for better sensitivity and larger mutation discrimination. Such advantages promise accurate genotyping results from our bead-based melting analysis with great potential for SNP genotyping scanning.

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REFERENCES


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