DETECTING GENETIC VARIATIONS IN A DROPLET
Yi Zhang1,3†, Dong Jin Shin1†, Tza-Huei (Jeff) Wang1,2,3,4,†
1Department of Biomedical Engineering, Johns Hopkins University, MD, USA
2Department of Mechanical Engineering, Johns Hopkins University, MD, USA
3Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, MD, USA
4Center of Cancer Nanotechnology Excellence at Johns Hopkins, Johns Hopkins University, MD, USA

ABSTRACT
We report a portable system for mutation detection using a droplet-based microfluidic platform. We demonstrate functionality of our device in analyzing mutations in mammalian cells by performing sample preparation in droplet followed by real time PCR and using melt curve apparatus for detection. Results suggest it is possible to perform melt curve assay from sample preparation to detection on a single platform.

KEYWORDS: droplet PCR, melt curve analysis, mutation scanning, lab-on-a-chip

INTRODUCTION
Early screening of genetic markers for diseases enables targeted treatment and improved prognosis, making a strong case for point-of-care diagnostics. We recently developed a droplet-based lab-on-a-chip platform which integrates cell lysis, DNA isolation and PCR on a single device for pathogen detection [1]. Our goal is to develop a point-of-care assay for genotyping using melt curve analysis. We present data in support of our system’s capability to perform sample preparation and melt curve analysis for genetic variation detection in mammalian cells.

THEROY
Silica superparamagnetic (SSP) beads are used in solid-phase DNA extraction. We utilize a microfluidic device that enables magnetic actuation of paramagnetic beads across multiple reagents pre-stored in the form droplets on the device. Droplet manipulation can only be achieved on surfaces with sufficiently low surface tension such that reagents do not disperse. Meanwhile, separation of magnetic beads from reagent requires sufficient surface tension to enable splitting. Our microfluidic platform utilizes channels formed by elevations to enable efficient separation of beads from droplet, while using Teflon® AF coating on the surface to enable droplet manipulation on chip.

DNA amplicons are interrogated using melt curve analysis, as shown in Figure 1. This technique measures the presence of double-stranded DNA (dsDNA) as a function of temperature, generally by measuring fluorescence intensity of sample mixed with dsDNA intercalating dyes. Melting profile of DNA is sensitive to parameters such as length, sequence and complementarity. In the case of genotyping, melt curve has been used in detection of heterozygous mutations [2]. Amplification of heterozygous gene segments result in presence of heteroduplexes, which are characterized by lower melting temperature than homoduplexes.

![Figure 1: Principle of mutation scanning using melt curve analysis. A) dsDNA intercalating dyes fluoresce with high intensity only when bound to duplex DNA. Denaturation results in a reverse sigmoidal curve as a function of temperature. B) DNA melt curve obtained from synthetic oligonucleotides. Heterozygous sample (red) is distinct from homozygote (black)](https://example.com/figure1.png)

EXPERIMENTAL
Microfluidic device: A device shown in Figure 2A (left) was created by curing PDMS inside a PTFE mold. The bottom surface of device was bonded to borosilicate glass and overlaid with mineral oil. A circular perforation of 4mm diameter was created in the droplet cradle indicated in Figure 2A in order to enable direct contact of droplet with borosilicate glass. The sample preparation process consisted of three stages: lysis, wash and elution. Five reagent droplets were dispensed on sample preparation device in following quantities: 21µL lysis buffer, 35µL wash buffer AW1, 2x25µL wash buffer AW2, 10µL elution buffer. Device operation procedure is shown in Figure 2B. 10µL cell suspension was dispensed into lysis buffer, followed by 0.5µL SSP beads. The beads were incubated in lysis and elution buffers for 10min, and in wash buffers for 30sec each. After each incubation step, beads were separated from the reagent by pulling through the gap between elevations.
**Figure 2: Droplet sample preparation platform. A) CAD render of device. The entire device is overlaid with mineral oil to provide bead mobility and prevent evaporation during PCR. Arrow indicates perforation. B) Sample preparation procedure. SSP beads are actuated in direction as indicated by arrows. C) A photograph of SSP bead separating from droplet.**

*Instrumentation:* Fluorescent signal was measured using epifluorescence arrangement consisting of a blue LED and photodiode, as shown in Figure 3B. Excitation source and detector were locked to 500±50Hz using in a phase sensitive configuration (Figure 3C) to enable measurement in ambient light. Temperature control was established using a 5cm x 5cm Peltier plate and a thermoelcetric controller unit (Accuthermo Tech). Peltier surface temperature was probed with a surface-mounted K-type thermocouple (Omega Engineering). Thermal cycling parameters were calibrated to the temperature attained at the contact between the cover slip and sample droplet. Mineral oil was used to provide thermal contact between droplet device and Peltier plate.

*Materials:* Pancreatic cell lines Panc-1 and Panc-10.05 and Panc-1 cell suspensions were obtained from Sidney Kimmel Comprehensive Cancer Center (Baltimore, MD). Each sample aliquot was diluted to approximately 5,000 cells per µL. SSP-based DNA purification kit (BioSprint 15 DNA Blood Kit, Qiagen) was used to extract genomic DNA from samples. Primers for PCR were as follows: 5’-taaggcctgctgaaaatgactg-3’ (forward) and 5’-tggtcctgcaccagtaatatgc-3’ (reverse) were used to generate 167bp amplicon [2]. LCGreen+ intercalating dye (Idaho Tech) was used to both monitor amplification in real time as well as to perform melt curve analysis.

**Figure 3: Apparatus overview. A) CAD render of melt curve apparatus. B) Device workflow. C) Optoelectronic circuit setup.**

**RESULTS AND DISCUSSION**

Amplification on chip: PID parameters and thermal cycling conditions were manually configured to provide rapid temperature cycling and stability at each stage. In order to test thermal cycling capability of our device, Panc-1 genomic extract was isolated on chip and amplified for 40 cycles. Figure 4 shows successful amplification on droplet platform in real time, as measured by an exponential increase in fluorescence during annealing stages over consecutive cycles. Melt curve was subsequently measured and found to match the profile of Panc-1 Kras target sequence with melting temperature of 77°C.

**Figure 4: DNA amplification in droplet. A) Real time PCR signal. B) Melt curve signal from product in Figure 4A.**
**Kras codon 12 mutation scanning in pancreatic cell lines:** After sample preparation on droplet platform, PCR was run with primer concentration of 0.4µM. Melt curve was measured using temperature ramp between 50°C and 100°C at a rate of 0.05°C/s. Figure 5A shows comparison of melt curves obtained from PCR amplicons of Panc-1 and Panc-10.05 lysates prepared in droplet.

**Melt curves synthetic targets:** In order to explore the possibility of using our platform for scanning other genetic variations, melt curve was measured using synthetic oligonucleotide to simulate amplicons of other genetic variations. We performed melt curve analyses on two synthetic targets: 110bp human beta-globin (HBB) gene segment and 60bp differentially methylated CDKN2B (p15) promoter segment from methylation-specific PCR (MSP) [4]. Figure 5B shows that mutations and differential methylation in synthetic targets can be detected using our droplet melt curve platform. Melt curve profiles for HBB profiles show a clear separation between three variants, with double mutation heterozygote clearly marked by double transition. p15 promoter melt curve profiles also show clear difference based on methylation status.

**CONCLUSION**
Preparation of mammalian sample and genotyping via melt curve analysis was demonstrated using our droplet sample preparation device and melt curve apparatus. With further improvements, it may be possible to explore detection of genetic variations.

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**REFERENCES**

**CONTACT**
*Tza-Huei (Jeff) Wang, email: thwang@jhu.edu
†These authors contributed equally.