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A droplet microfluidic approach to single-stream nucleic acid isolation and mutation detection

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Abstract

In this work, a droplet microfluidic platform for genetic mutation detection from crude biosample is described. Single-stream integration of nucleic acid isolation and amplification is realized on a simple fluidic cartridge. Subsequent DNA melting curve is employed with signal normalizing algorithm to differentiate heterozygous K-ras codon 12 c.25G>A mutant from the wildtype. This technique showcases an alternative to modular bench-top approaches for genetic mutation screening, which is of interest to decentralized diagnostic platforms.

Keywords

Droplet microfluidics; Droplet PCR; Melting curve analysis; Mutation detection

1. Introduction

Genetic testing is a diverse and growing field with applications ranging from diagnostics, newborn screening, and forensics. However, the utility of genetic testing in clinical setting is confined to those with access to core facilities specializing in molecular diagnostic assays. In light of this situation, recent development in lab-on-chip systems have demonstrated miniaturized platforms capable of isolating nucleic acids and amplifying biomarkers of interest using polymerase chain reaction (PCR). [1–3] Nonetheless, fluidic management

remains an obstacle to practical deployment of platforms utilizing microchannels, due to the complexities of fluidic handling which requires intricate microfluidic architecture designs as well as external fluidic interface. [4, 5] To address these challenges, platforms utilizing discrete droplets for liquid control have emerged, enabling pumpless and valveless handling of reagents. In this digital microfluidics (DMF) paradigm, droplets are actuated using magnetic particles, [6–9] dielectrophoresis [10] and electrowetting-on-dielectric (EWOD) [11, 12] to perform all essential fluidic manipulations for bioanalysis, including transport, mixing, splitting, and merging of reagents. Such platforms enable efficient handling of samples and reagents with much simplified fluidic management.

As a convenient means of analyzing genetic variations, melting curve analysis has recently emerged as a potential alternative to sequencing [13] or mass spectrometry [14] for genotyping applications. This technique utilizes differences in thermodynamic properties of oligonucleotides to resolve subtle differences in nucleotide sequence. The dissociation characteristics of double stranded DNA during heating can be monitored via the use of DNA intercalating dyes. [15] By monitoring the fraction of double stranded DNA over a positive thermal ramp, thermodynamic properties associated with the sequence are resolved as a function of temperature. Because the stability of DNA double helix is a function of base composition and length, DNA samples containing single nucleotide polymorphisms (SNPs) generate melting profiles that are distinct from their wild-type counterparts. Melting curve analysis benefits from its simple format for genotyping, requiring only a single universal primer pair for product amplification and no additional probes for allelic discrimination, in contrast with other PCR-based genotyping assays such as allele-specific PCR [16] and allele-specific oligonucleotide probes [17].

This communication presents an integrated microfluidic melting curve analysis platform capable of identifying genetic mutation from complex biological samples such as whole blood. Firstly, extraction and purification of nucleic acids from crude biological samples is achieved using a magnetic bead-based actuation mechanism. [8] Silica-coated magnetic particles are used as a solid phase for DNA extraction and transport, while topographical features raised on the surface of the microchip enable efficient splitting and confinement of reagents. Following DNA extraction, a miniaturized thermal cycling and detection module performs real-time amplification and melting curve acquisition directly from the microchip. Melting curve profiles were used to demonstrate genotyping capability for heterozygous mutation in K-ras oncogene. This study describes the first integrated droplet microfluidic platform applied to genotyping with melting curve from crude biological samples.

2. Experimental

2.1 Microfluidic device and instrumentation

The microfluidic device (Fig. 1a) was fabricated by casting polydimethylsiloxane (PDMS) pre-polymer in a computer numerical control (CNC) machined polytetrafluoroethylene (PTFE) mold and curing at 80°C for 60 minutes (Fig. S1). This was followed by puncturing a 4 mm aperture in the PCR reagent compartment and bonding with a 100 µm-thick glass coverslip via oxygen plasma activation. Afterwards, the device surface was rendered hydrophobic by dip-coating in 1% Teflon AF 1600 (DuPont, USA). The overall cartridge

dimension was 35×43 mm with six identical compartments. The melting curve instrument was a miniaturized epifluorescence detector combined with a PID-controlled thermoelectric element for simultaneous monitoring of fluorescence and temperature (Fig. S2). Temperature calibration was achieved by adjusting PID setpoints while monitoring PCR chamber temperature with a secondary thermistor to obtain the desired temperatures on chip (Fig S3). Raw melting curve signals were processed using custom LabVIEW program which implemented curve normalization via exponential background subtraction. [18]

2.2 Cartridge priming and assay protocol

The proposed device integrates genomic DNA extraction, real-time PCR, and melting curve analysis using silica-coated magnetic beads as a mobile solid phase extractor. The device was pre-primed with reagents in the form of sessile droplet in the following order: 21.5 μ L lysis/binding buffer (10 μ L Buffer AL, 10 μ L isopropyl alcohol, 1 μ L serine protease, and 0.5 μ L MagAttract Suspension G), 35 μ L wash buffer 1, 30 μ L wash buffer 2, another 30 μ L wash buffer 2, and 25 μ L PCR mixture (1× LCGreen+ dye, 1× PCR buffer, 3.5 mM MgCl₂). All reagents were dispensed into the cartridge using pipettes before the experiment. All droplets were overlaid with mineral oil to prevent evaporation, resulting in aqueous reagent droplets sealed in a continuous oil phase. Lysis/binding and wash buffers were components from commercial DNA isolation kit (BioSprint Kit, QIAGEN). For each extraction process, biological sample was first injected into the lysis buffer droplet and incubated for 10 minutes. During this process, nucleus-containing cells in sample matrix were lysed to release the genomic material. The released genomic DNA then adsorbed to silica-coated surface of magnetic beads in the presence of chaotropic salt in the lysis/binding buffer. [19] After incubation, DNA adsorbed on magnetic beads were transported from the lysis buffer using a permanent magnet and rinsed in a series of washing buffers to remove chaotropes and PCR inhibitors, followed by elution in PCR mixture for 10 minutes at 70°C. The condition of PCR buffer favoured the release of DNA from the silica surface. Lastly, the beads were split from the PCR mixture into the waste chamber, and the device was loaded onto the miniaturized instrument for amplification and melting curve analysis.

Droplet operations were facilitated by the use of topographical barriers, which serves a dual purpose of confining aqueous reagents in isolation and enabling splitting of magnetic particles from the aqueous phase. Topographical barriers in the current work were 5×5 mm chambers with ~5 mm height and 1 mm vertical gaps on either side of the chamber. Using a manually operated neodymium magnet positioned ~1 mm below the bottom surface of the cartridge, magnetic particles were removed from the original aqueous phase and available for transport into subsequent reagent solution. This process replaces liquid aspiration and addition with a transport of magnetic solid phase between two buffer systems. The principle of droplet splitting by using topographical barriers is further explored in [8]. During washing and incubation steps, magnet is removed from the device. As the bead surface is silica-coated and therefore hydrophilic, the beads remained compartmentalized in aqueous buffer phase rather than the surrounding oil phase.

3. Results and Discussion

To demonstrate genomic DNA extraction and amplification using the microchip, we first characterized DNA extraction by performing quantitative PCR from the extracted genomic DNA. Stock of confluent Panc 10.05 cells suspended in 1 mL phosphate buffered saline was serially diluted and quantified using a hemocytometer slide. Afterwards, 10 μ L samples were loaded into a lysis mixture and processed on the microchip. In this case, the PCR mixture was replaced with 10 μ L elution buffer (BioSprint Kit, QIAGEN), and the isolated DNA was quantified using PicoGreen assay kit (Invitrogen, CA). As shown in Figure 2a, the DNA yields are linearly proportional to the amount of cells in each dilution. Using a theoretical genome mass of 6.6 pg for each diploid cell, the DNA yield fraction was found to range between 4 to 11% (Figure 2b). The modest extraction yield can be attributed to reduced lysing efficiency due to the lack of incubation at elevated temperatures. Since the white blood cell count is on the order of 10^3 – 10^4 per μ L for normal adults, [20] the extraction yield achieved by this platform is sufficient to capture target genome for amplification.

Next, we performed DNA extraction, amplification, and melting curve analysis on chip with crude biological sample to demonstrate genotyping capability of the proposed platform. 10 μ L unspun whole blood from male donor with heparin anticoagulant (Biological Specialty Corp, USA) was processed on the microchip using steps described in Figure 1a. Following genomic DNA isolation, the chip was inserted into the custom instrument for amplification. 167-bp products were amplified using primers listed in Table 1, as described previously. [21] Cycling parameters included a 10-minute thermal activation at 95°C, followed by cycling between 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute for 40 cycles. Figure 3a shows the real-time fluorescence signals obtained during the anneal phase of each cycle, with a threshold cycle number of 28. At the end of thermal cycling, the sample was cooled to 25°C for 3 minutes, followed by holding at 50°C for another 3 minutes. A positive thermal ramp was applied up to 95°C at a rate of 0.05°C per second. The resulting raw melting curve signal is presented in Figure 3b. In order to convert fluorescence signal into double-stranded DNA fraction, raw data was normalized using background subtraction method. [18] Background fluorescence intensity is modelled as an exponentially decaying function of temperature. Fitting an exponential curve to the post-melt region and subtracting the curve from raw signal generates a sigmoidal profile that is constant at pre-melt and post-melt regions. Normalization of data between zero and unity generates the final curve as shown in Figure 3c.

Wild-type genome from a healthy human blood sample was differentiated from the mutant cell line genome using the droplet platform. The adenocarcinoma cell line Panc 10.05 has a heterozygous mutation in K-ras codon 12 (c.35G>A). Sequence information from amplified products was verified by Sanger sequencing using Applied Biosystems 3730xl DNA analyzer (Fig. 4b). As shown in Figure 4a, melting profile generated from wild-type genome is clearly differentiable from the heterozygous mutant. The lower melting point of the heterozygote is attributed to formation of heteroduplex DNA between wildtype and mutant strands, which are less thermally stable than fully complementary DNA. [22] It should be noted that homozygous mutants will generate fully complementary duplexes, whose

thermodynamic properties are very similar to wildtype duplexes and are consequently more challenging to differentiate at a single nucleotide resolution. Melting curve analysis is generally conducted with shorter PCR amplicons (<200bp) as described in this work. The use of melting profiles in addition to melting temperature during analysis helps to differentiate between PCR products whose amplicon lengths and base pair compositions are similar.

Single-stream assay as described in this work refers to the streamlining of fluidic processing steps such that the entire assay can be performed by a unified control mechanism. This paradigm is in contrast to the conventional bench-top process where reagent handling steps such as aspirating, addition and mixing are required. In the droplet microfluidic approach, a mobile solid phase-based assay protocol obviates challenging liquid handling steps by replacing the liquid transfer steps with a magnet-based transport of the solid phase across a sequence of liquid reagents. Magnetic droplet manipulation in this work was performed manually with a permanent magnet, although it should be noted that automated operation can be achieved through the use of a translation stage or an electromagnetic coil array as described previously [23].

This work expands significantly on our previous work on PCR-based diagnostic platforms [3, 8] by incorporating melting curve analysis for genotyping application. The additional qualitative information that can be derived from melting curve analysis provides a distinct advantage over previous amplification-based detection method where only binary information can be obtained for each primer pair. Within the context of broader literature, melting curve analysis of pre-prepared samples from a device fabrication perspective has been recently explored in a lab-on-a-foil device [24]. The current work approaches the use of melting curve analysis from a broader scope in combination with sample preparation and amplification, facilitated by magnet-based droplet manipulation. Due to the versatility of melting curve techniques, it is anticipated that an increasing number of novel applications will emerge at the intersection between melting curve analysis and microfluidics.

4. Conclusion

This report demonstrates a simple droplet microfluidic platform for performing single-stream genotyping assay via melt curve analysis. The platform is capable of processing crude biological samples such as whole blood. Combined with instrumentation capable of thermal ramp and a suitable normalization algorithm, genetic polymorphisms could be identified in PCR amplicons from biological samples. Due to its simple operation and portability, the platform presents a possible direction for designing decentralized platforms dedicated to mutation scanning from a small aliquot of patient blood sample.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

a) Overview of droplet operation for whole blood processing. Black dashed lines represent the path of magnetic beads. 1) Sample matrix contains red and white blood cells, of which only the white blood cells contain genomic material; 2) Lysis buffer breaks down cells to release genomic DNA, which binds to the silica surface of magnetic beads. 3) Magnetic beads are merged with a series of wash buffers where residual debris from lysis is removed from beads; 4) Washed beads are merged with PCR droplet where DNA is eluted. b) PCR droplet from the end of 1a is processed through two stages of thermal modulation. The first stage is thermal cycling, which amplifies a segment of DNA specific to primers contained inside the droplet. The second stage generates a positive temperature ramp, at a rate of 0.05°C per second, for melting curve analysis. As duplex DNA begins to denature, dyes are dissociated from denatured bases and strongly quenched in free solution, resulting in a sharp decline in signal.

Fig. 2.

Characterization of DNA extraction performance on droplet platform. Dilutions of Panc 10.05 cell cultures were processed on the microchip and the extracted DNA was quantified using PicoGreen assay (Quant-iT, Invitrogen). The extracted DNA was analyzed as a function of a) cell input and b) fraction yield based on 6.6 pg DNA per cell.

Fig. 3.

On-chip amplification and melting from crude biological sample. Genomic DNA was purified from 10 μ L human whole blood on the microchip and eluted in the PCR mixture. The microchip was mounted on the miniaturized instrument for thermal cycling and melting curve acquisition. a) Amplification enters exponential phase at around 28 cycles. b) Raw melting profile obtained after amplification in 3a. c) Data from 3b is processed using custom LabVIEW software to generate a normalized melting profile. Raw signal was processed through Savitsky-Golay filter with a window of 1°C prior to normalization.

Fig. 4. Melting curve analysis for K-ras codon 12 mutation. Adenocarcinoma cell line Panc 10.05 carries a known heterozygous mutation c.25G>A. a) Normalized melting profiles from the wildtype and the mutant cell-line genome amplicons. Inset in upper right corner is a derivative plot with dashed lines indicating average melting temperatures, defined as the peak of derivative plot. Approximately 1°C difference is observed between the wildtype and heterozygous mutant. b) Genotype of both amplicon set were verified by Sanger sequencing. Panc 10.05 displays a mixture of guanine and adenine signals at the mutated base.

Table 1

Primer sequences

Oligonucleotide name	Sequence
K-ras forward	5'- TAAGGCCTGCTGAAAATGACTG -3'
K-ras reverse	5'- TGGTCCTGCACCAGTAATATGC -3'