ONE-STEP DIGITAL PLASMA SEPARATION FOR MOLECULAR DIAGNOSTICS
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ABSTRACT
Current blood-based quantitative nucleic acid (NA) detection requires many sample preparation steps. We have developed the digital plasma separation platform, which combines autonomous parallel plasma separation and sample compartmentalization for digital NA amplification in one-step. For NA detection we utilized isothermal Recombinase Polymerase Amplification (RPA). Simple fluorescence endpoint counts of positive wells can be done by a standard microscope, making it possible to be an alternative low cost solution compared to RT-PCR. We demonstrated digital amplification from spiked samples and the detection of Methicillin-Resistant Staphylococcus Aureus (MRSA) DNA directly from blood samples in 30 min with DPS.

KEYWORDS: Sample preparation, plasma separation, digital amplification, isothermal amplification, RT-PCR, Recombinase Polymerase Amplification, MRSA

INTRODUCTION
The standard protocol for blood based nucleic acid testing (e.g. RT-PCR) requires an initial sample purification step that involves extensive manual handling. It remains a challenge to integrate sample preparation into the same device in a one-step manner. Furthermore, real-time PCR requires 2-6 hours of assay time, trained technicians, centralized labs, or costly equipment for testing. There is a need to consolidate and automate sample preparation with NA amplification readout to shorten the assay time and steps required. We have found that more recent isothermal amplification methods, such as RPA can amplify directly in plasma samples [1]; therefore, we focused on developing a plasma separation system that is integrated with NA detection. Other work with plasma separation [2], such as filter/bead based separation, inertia based separation, Zweifach-Fung effect, or accoustophoresis, do not integrate the concept of digital detection, and often require peripheral equipment such as external pumps and power sources to actuate the system. On the other hand, other digital amplification examples [3] do not integrate sample preparation with readout. Of the above mentioned techniques, even if there were NA detection demonstrated, it was mostly done with NA spiked in pure water; detection from whole blood samples directly have not been demonstrated. Here we present a method utilizing inertial based plasma separation techniques to extract pathogenic nucleic acid from whole blood samples for nucleic acid testing in one-step. Our system has the advantages of not causing hemolysis nor clogging, which is a common problem with membrane or bead based separation methods. The liquid loading is via passive PDMS degas pumping [4], therefor no external pumps and power source is needed. Here we demonstrate that digital plasma separation can integrate sample preparation with digital isothermal amplification using RPA, and detect of MRSA DNA directly from human whole blood samples in 30 minutes. To our best knowledge, this is the first time demonstrating the coupling of digital plasma separation with isothermal NA detection for direct NA detection from blood samples.

THEORY
Using a “microcliff” structure, we take advantage of blood cell sedimentation, and skim plasma into wells. By separating the red blood cells, we ensure that there is less optical obstruction of the fluorescence signal, and less enzymatic interference of the polymerase due to hemoglobin inhibition. Hemoglobin is a well-known PCR inhibitor as the chelating properties disrupts the ion concentrations in the sample [5] and thus inhibits polymerase activity. Since there is very low shear stress created on the RBCS, there is minimal hemolysis. The DPS design also avoids blood cells clogging since there are no features that would cause blood cell stacking against the flow. DPS enables digital NA amplification assays (RPA) to be performed directly from the separated plasma. We arranged the microcliff structures in an array; therefor large numbers (200~1500) of wells can be processed in parallel. Samples are then digitized by an air plug automatically by degas flow, and the RPA reaction is initiated in each well to commence digital amplification (Figure 1, 3).

EXPERIMENTAL
Microfluidic chips were fabricated with the standard soft lithography processes. PDMS was cast onto SU8 patterned wafers. Blood and RPA reagents were mixed prior to loading. 100µl of blood samples mixed with RPA reagents (RPA exo kit, Twistdx) were loaded each time. Partial components of RPA mix were lyophilized into the wells. Devices were stored in house vacuum (~70kPa) overnight before loading samples. Samples can be sealed in vacuum pouches and still be fully functional at least up to a year. After the samples loaded and compartmentalized, the chip was put in a 37°C incubator for RPA incubation. After incubation, fluorescence signal was detected by a fluorescence microscope (Zeiss, Axiozoom).
RESULTS AND DISCUSSION

Plasma separation efficiency was >99% when flow rates were lower than 100µm/s into the wells. One-step plasma separation and sample compartmentalization was possible in ~10 minutes; 200–1500 wells (30–100nl/well) can be separated from whole blood mixed with RPA reagents (100µl) (Figure 1). The user only needs to drop the blood/RPA mix onto the chip for plasma separation and digitization to commence. Degas loading is stable up to 30 minutes. MRSA DNA spiked in whole blood was detected within 30mins with RPA in one-step with the DPS method (Figure 2). No hemolysis or clogging was observed in the devices. We also tested digital amplification with DPS by spiking MRSA DNA in water, the dynamic range of digital detection was 10³~10⁶ copies/ml, which matched well with theory (Figure 3). We are currently testing the DPS design with other infectious disease models.

Figure 1: Digital Plasma Separation enables one-step digital amplification by combining parallel plasma separation and sample compartmentalization. (A) Detailed view of the “Microcliff” plasma skimming structure. The working principle of separation is based on inertia and sedimentation. (B) The user simply drops blood onto the chip and flow starts automatically by passive degas driven flow, no external pumps are needed. Plasma gets separated and compartmentalized automatically by the Digital Plasma Separation design. 200–1500 wells (30–100nl) can be loaded within 10 minutes. (C) By counting positive wells at the end point of RPA amplification with fluorescence imaging, the template concentration can be quantified, eliminating the necessity of costly real-time imaging.
CONCLUSION

The one-step digital plasma separation platform can be applied to detect nucleic acids in whole blood under ~30 mins. Digital NA amplification was demonstrated to be compatible with this system. This is a significant improvement compared to current costly commercial systems, which can take hours of assay time, require trained technicians, and need costly equipment in centralized labs. We envision this portable technology to be a new paradigm for low cost point-of-care blood-based quantitative NA assays in low resource settings such as Africa.

ACKNOWLEDGEMENTS

The authors acknowledge the Bill & Melinda Gates Foundation (Global Health Grant: OPP1028785) for funding this work. The authors also would like to thank Chi-cheng Fu for extensive feedback and discussion on this work. And also Lucy Hu, Rita Huang, and Qiong Pan for assistance in chip fabrication.

REFERENCES


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