QUANTIFICATION OF HIV AND HCV VIRAL LOAD WITH LARGE DYNAMIC RANGE USING MULTIVOLUME DIGITAL REVERSE TRANSCRIPTION PCR ON A ROTATIONAL SLIPCHIP

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ABSTRACT

This presentation describes a SlipChip-based microfluidic platform capable of performing multiplex digital RT-PCR with large and tunable dynamic range, using a mathematical approach based on Most Probable Number theory. By using compartments of different volumes, we achieved a dynamic range of 520 – 4,000,000 molecules/mL at 3-fold resolution with 95% confidence interval. This SlipChip platform enables fast, accurate, low-cost, "instrument-free" quantification of RNA, crucial for resource-limited settings. We validated the device using deidentified clinical patient HIV viral RNA and results from the SlipChip were in good agreement with Roche COBAS® AmpliPrep / COBAS® TaqMan® HIV-1 Test, v2.0 system. We also expanded the device to allow for multiplexing and validated the multiplexed device using HIV and HCV viral RNA.

KEY WORDS Digital RT-PCR, High dynamic range, multivolume, HIV viral load

INTRODUCTION

This presentation will describe a recently developed method[1] to quantify RNA using digital RT-PCR on a multivolume (MV) SlipChip platform. The HIV viral load test is a quantitative measurement of HIV RNA which provides important information in monitoring disease status and in guiding therapy. As antiretroviral treatments become more widely available, there is an ever-increasing demand to evaluate viral loads at regular intervals to prevent the spread of drug resistance. However, it remains difficult to do, particularly in resource-limited settings, because it requires the measurement of RNA over a large dynamic range (from 50 to 10^6 molecules/mL for HIV and up to 10^8 molecules/mL for HCV).

The most commonly used method to quantify RNA is real-time quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR), but it is usually only provided in centralized facilities with multiple instruments and highly skilled technicians, thus its application is not accessible in resource-limited settings. Compared with real-time RT-PCR, digital RT-PCR developed on microfluidic platforms has certain advantages such as low cost and small sample volume. However most of these microfluidic platforms still have two drawbacks: the need for relatively complex control systems to handle samples and the need for large numbers of experiments/compartments to obtain wide dynamic range and high resolution.

To solve this problem, a rotational SlipChip with sets of compartments of different volumes was developed (Figure 1). The SlipChip is a microfluidic platform consisting of two plates that can manipulate liquid samples from pL-to µL-scales by relative movement of the plates without the need for complex control systems[2]. The SlipChip has been previously used for multiplex PCR[3], digital PCR[4], and digital isothermal amplification (RPA)[5]. Instead of the uniform well volumes of previous designs, this MV design used four sets of wells with volumes of 1 nL, 5 nL, 25 nL, and 125 nL respectively. The volume of the well determines the probability that molecules will be trapped in the well. When there is at least one molecule present, amplification generates a "yes" signal, otherwise a "no" signal will be observed. We demonstrated the quantification of HIV in archived, deidentified clinical patient samples with digital RT-PCR on this design. We then modified the design to incorporate multiplexing capability while maintaining the large dynamic range by adding additional well volumes. The multiplexed design could analyze five separate samples simultaneously; when this second design is used to analyze a single sample, it has the potential to reach an even higher dynamic range (40-20,000,000 molecules/mL)[1].

EXPERIMENTAL

The procedure for fabricating the SlipChip was described in previous work[2]. The SlipChip was made from soda−lime glass plates coated with chromium and photoresist. By using a two-step exposure and etching procedure, patterns of two different depths were generated on the chip. After etching, the glass plate was silanized with dichlorodimethylsilane and assembled under 4:1v/v tetradecane: mineral oil under the stereomicroscope for precise alignment. A control RNA molecule (906 nucleotide) was synthesized from the LITMUS 28iMal Control Plasmid using a HiScribe™ T7 In Vitro Transcription Kit and purified using MinElute PCR purification. Plasma samples containing the HIV virus were obtained from archived, deidentified patient samples at the University of Chicago Hospital. Plasma containing a control HCV virus (25 million IU/mL) was purchased from AcroMetrix (Benicia, CA). All plasma samples were purified using iPrepTM purification. Primers for the control RNA (906 nt) were: GAA GAG TTG GCG AAA GAT CCA CG and CGA GCT CGA ATT AGT CTG CGC.

Primer sequences for HIV viral RNA was selected from a previous publication[6]: GRA ACC CAC TGC TTA ASS CTC AA; GAG GGA TCT CTA GNY ACC AGA GT. Primer sequences for HCV viral RNA were selected from a previous publication[7]: GAG TAG TGT TGG GTC GCG AA; GTG CAC GGT CTA CGA GAC CTC. The amplification was performed using a PCR mastercycler machine (Eppendorf). All fluorescence images were acquired using Leica DMI 6000 B epi-fluorescence microscope with a 5X / 0.15 NA objective and L5 filter at room temperature and stitched together using MetaMorph software (Molecular Devices, Sunnyvale, CA).

RESULTS AND DISCUSSION

The theory for design and analysis of this MV SlipChip was based on Most Probable Number (MPN) theory[8], and is described in detail in an accompanying study[9]. The analysis combined the results from each volume (i is from1 to 4) using Equation 1 to solve for the concentration of target moleculese in the sample, λ (molecules/mL). n_i is the total number of wells at each volume i, b_i is the number of negative wells at that volume, and v_i is the well volume (mL). By combining the results from each volume, the "most probable" concentration can be more precisely identified, and also the confidence interval is improved. The confidence interval is ascertained by determining the standard deviation, σ, for ln (λ)using Equation 2, which was derived based on the Fisher information[8-9].

$$\sum_{i=1}^{m} n_i \cdot v_i = \sum_{i=1}^{m} \frac{(n_i - b_i)\cdot v_i}{\left(1 - e^{-v_i \cdot \lambda}\right)} \qquad (1)$$

$$\sigma = \frac{1}{\sqrt{\lambda^2 \cdot \sum \frac{{v_i}^2 \cdot n_i}{e^{v_i \lambda} - 1}}} \qquad (2)$$

We first validated the performance of the MV SlipChip by running digital RT-PCR using a 6 order-of-magnitude serial dilution of the control RNA (906 nt). The results showed good agreement between the experimental value and expected value (data not shown). To further validate the feasibility of using a rotational MV SlipChip to quantify HIV viral load, we used it to measure HIV viral RNA purified from archived samples of HIV-infected blood plasma from two different deidentified patients. The purified HIV viral RNA from each deidentified patient sample was serially diluted and characterized by MV digital RT-PCR on the SlipChip and each experiment was repeated at least four times. The same plasma samples were characterized using the Roche COBAS® AmpliPrep / COBAS® TaqMan® HIV-1 Test, v2.0 (CA/CTM v2.0) according to the manufacturer's recommendation in a single experiment and the resulting concentrations were treated as the standard for characterization. For both samples, the data from the SlipChip were self-consistent and in agreement with the expected values from the Roche measurement (Figure 2). When considering the effect of concentration during sample preparation, the lowest concentration of serially diluted HIV viral RNA detected on the SlipChip corresponded to 36 molecules/mL in the patient plasma, and the highest concentration detected corresponded to 1.7 million molecules/mL in the patient plasma.

Figure 1. Bright field image of the rotational SlipChip after slipping to form isolated compartments, shown next to a U.S. quarter.

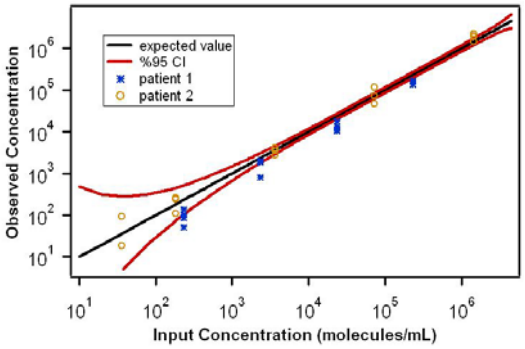

Figure 2. MV digital RT-PCR results for quantification of HIV viral load in two archived deidentified patients' samples.

We also incorporated multiplexing into the system while maintaining the dynamic range by adding two additional sets of wells (0.2 nL and 625 nL, respectively) and splitting the device into five sections, as shown in Figure 3A. After thermal cycling, no false positives were observed in either negative control for HIV or HCV and no cross-contamination was observed among different samples (Figure 3B). If this SlipChip design is used for a single sample instead of five individual samples, the dynamic range of the device with 3-fold resolution is increased to 173 to 19,900,000 molecules/mL with lower detection limit of 40 molecules/mL. Even if sample preparation provides only a modest concentrating effect, this device would enable detecting targets at concentrations of 10-20 molecules/mL in the original sample.

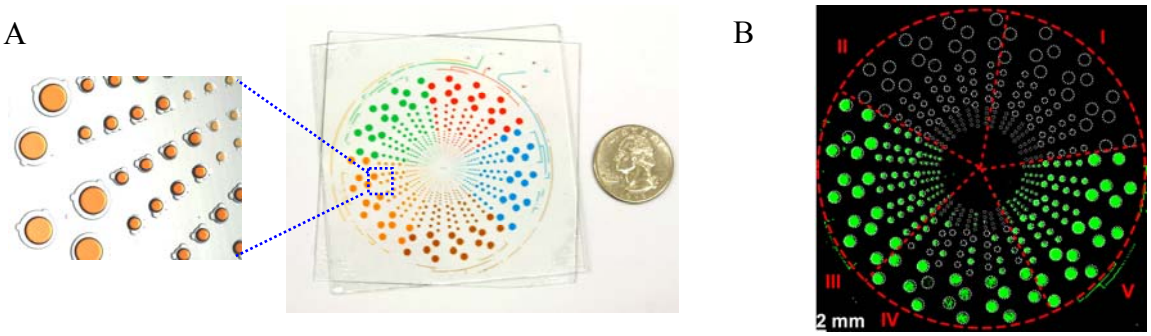

Figure 3. A SlipChip for multiplexed MV digital PCR with high dynamic range. A) A photograph of a multiplex device for up to 5 samples with a total of 80 wells of 625 nL, 160 wells of 125 nL, 160 wells of 25 nL, 160 wells of 5 nL, 160 wells of 1 nL, and 160 wells of 0.2 nL. B) Fluorescent photograph of a multiplexed digital RT-PCR detection panel: I) negative control for HCV (HCV primers with no loaded HCV RNA template); II) negative control for HIV (HIV primers with no loaded HIV RNA template); III) HCV viral RNA measurement; IV) internal control with purified 906nt RNA template; V) HIV viral RNA measurement.

CONCLUSION

Here we demonstrated the use of a rotational MV digital SlipChip for quantification of RNA over a large dynamic range. The first design was characterized using both synthetic HIV RNA and HIV RNA purified from archived deidentified clinical patient samples. The results showed mutual consistency between different volumes and agreements with theoretical predictions. Multiplexing was successfully integrated into the second design, and we showed the potential of using this design to achieve an even higher dynamic range. This MV digital SlipChip is valuable for a number of applications including viral load, rare cells and mutations detection, prenatal diagnostics and monitoring residual disease.

ACKNOWLEDGEMENT

We thank Heidi Park for contributions to writing and editing this manuscript. This work was supported by NIH Grant No. 1R01 EB012946 administered by the National Institute of Biomedical Imaging and Bioengineering and by the NIH Director's Pioneer Award program, part of the NIH Roadmap for Medical Research (1 DP1 OD003584).

Disclosure: F.S. and R.F.I have a financial interest in SlipChip, LLC.

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