SINGLE-MOLECULE COUNTING WITH MICROFLUIDICS, DIGITAL ISOTHERMAL AMPLIFICATION, AND A MOBILE PHONE IS MORE ROBUST THAN KINETIC BASED REAL-TIME QUANTIFICATION

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ABSTRACT

This paper shows, using purified HIV RNA, that nucleic acid quantification in a digital format with reverse-transcription loop-mediated isothermal amplification (dRT-LAMP) is more robust than the standard real-time assay. Specifically, we show that the quantitative outcome of a dRT-LAMP reaction is insensitive to temperature changes over a 6 °C range, and that dRT-LAMP reactions can be imaged using a cell phone, with the resultant images being suitable for automatic analysis and quantification. We have previously shown success in quantification of HIV RNA with dRT-LAMP [1], with that study focused specifically on improving reaction efficiency. We have also previously shown, using the digital recombinase polymerase amplification, that digital reactions can be temperature tolerant [2]; here we extend this previous work by directly comparing digital vs. real time performance, as well as adding further experimental perturbations of non-quantitative imaging and automatic analysis.

KEYWORDS: Digital amplification, Loop-mediated isothermal amplification, Cell phone, Automated analysis

INTRODUCTION

Quantitative diagnostic assays are required in many situations, including HIV monitoring and treatment. Currently the standard techniques for quantifying nucleic acids such as HIV are based on real-time non-linear amplification assays. In a real-time assay, the progress of a reaction is monitored, yielding a reaction trace. The concentration of an unknown sample can be determined by comparing its reaction trace to those of known concentrations. This process relies on precise and sensitive monitoring of a series of amplifications proceeding at a constant kinetic rate; any perturbations in the kinetic rate or reaction monitoring would alter the reaction trace and an incorrect concentration would be calculated. The issue of constant amplification kinetics is especially significant in non-linear amplification assays, such as LAMP, because small changes in the kinetic rate have an exponential effect on the final outcome. This can especially be a problem in HIV quantification where a small 3-fold change in concentration is considered clinically significant [3].

While real-time assays are suitable for a centralized laboratory with environmental controls, refrigerated reagent storage, trained personnel, and specialized equipment, they are not sufficiently robust for usage in limited resource setting (LRS) where these conditions are not always achievable. We hypothesized that a more suitable quantitative analysis format for LRS would be the digital format. In a digital format, a sample is separated into discrete volumes such that not all volumes contain the analyte of interest. Therefore, a qualitative end-point analysis can be performed on each individual reaction volume, and Poisson statistics can be used to calculate an original concentration. As long as a single molecule can be amplified into an observable signal within a specified time frame, the rate at which the amplification proceeded does not need to be known. In addition, due to the "on/off" nature of each reaction volume, semi-quantitative imaging devices can be used as long as they are capable of differentiating between these two states.

LAMP was specifically chosen for this analysis as it is validated as a real-time assay [4], compatible with the digital format [1,5], compatible with the highly fluorescent calcein chemistry [6], and compatible with chemical heaters which are ideal for LRS [7]. We used the SlipChip [8] architecture for performing the digital assays.

EXPERIMENTAL

Concentrations of $1x10^5$ and $2x10^5$ copies/mL were evaluated for both the real-time and digital formats so as to

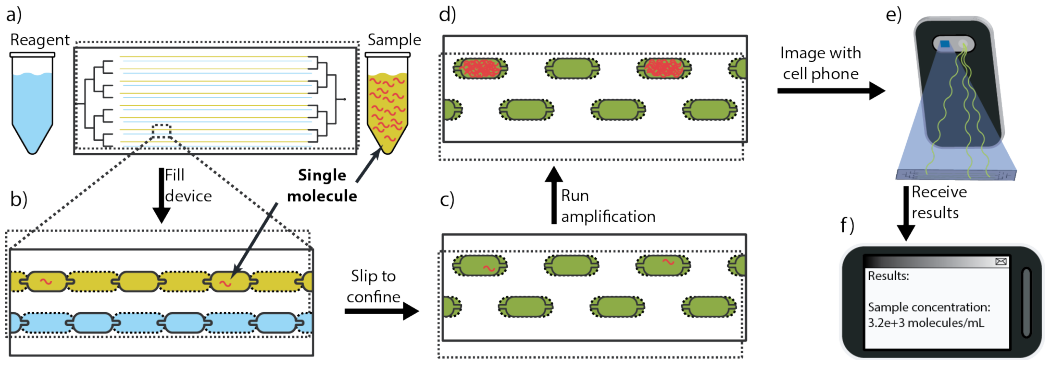

Figure 1: A schematic drawing illustrating the experimental procedure. a,b) the SlipChip is filled. c) Slipping discretizes volumes. d) Amplification is performed. e) The device is imaged with a cell phone. f) The automatically analyzed results are received via email.

determine the resolution of the assays. Real-time RT-LAMP experiments were performed on an Eco real-time PCR thermocycler. Real-time and digital experiments were performed at 57, 60, and 63 °C to evaluate the robustness to changes in the amplification rate. Digital experiments were imaged using a Leica DMI-6000 microscope as a control, and a modified Nokia 808 pureview cell phone for simulating non-quantitative imaging. The Nokia cell phone, which was used for both excitation and collection of fluorescent data, was modified by first placing two-halves of a dichroic filter (FD1B, Thorlabs) over the camera flash for providing the excitation light, and second by placing a non-branded 0.67 x wide field lens modified with a long-pass filter (5CGA-530, Newport) over the camera lens for collecting excitation light. To test the fidelity of the collected cell-phone images, an automatic processing algorithm was developed to analyze the cell-phone images. The cell-phone processing algorithm makes use of the "cloud" by automatically transmitting collected images to a remotely located server where they are automatically analyzed, with the user receiving the results of the analysis through email. The overall digital experimental workflow can be seen in Figure 1.

RESULTS AND DISCUSSION

Real-time RT-LAMP experiments could successfully distinguish a two-fold concentration change between $1x10^5$ and $2x10^5$ copies/mL of HIV-1 RNA at each individual temperature (57 °C p=0.07, 60 °C p=0.01, 63 °C p=0.04); however, when the data from the different temperatures was pooled, the concentration change could no longer be distinguished (p=0.25) (Figure 2a). Experiments performed using the dRT-LAMP method imaged with the microscope showed that not only could a two-fold concentration change be distinguished at each temperature (57 °C p=0.03, 60 °C p=0.02, 63 °C p=0.02), but this ability to distinguish this two-fold concentration change was maintained when the data from the different temperatures was pooled ($p= 6.7x10^{-7}$) (Figure 2b).

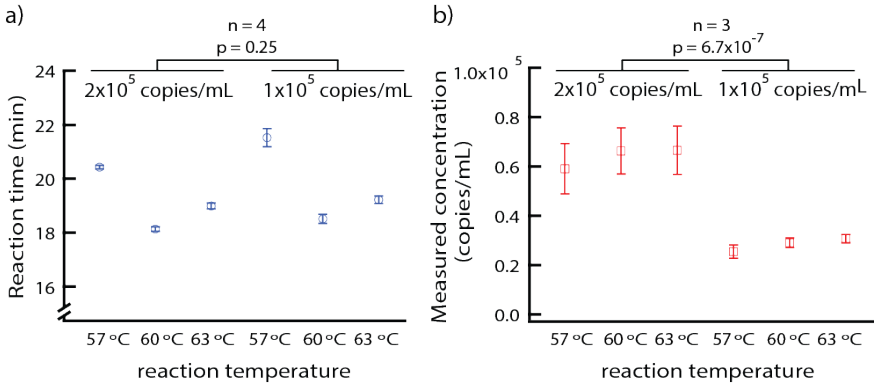

Figure 2: A comparison of real-time LAMP and dRT-LAMP performed at 57 °C, 60 °C, and 63 °C with concentrations of $1x10^5$ and $2x10^5$ copies of RNA/mL. a) The results of real-time lamp experiments as a plot of reaction time vs. reaction temperature. No statistical significance can be observed between the two concentrations. b) The results of dRT-LAMP experiments as a plot of measured concentration vs. reaction temperature. Statistical difference can be observed between the two concentrations.

The dRT-LAMP reactions were imaged by the cell phone under two different conditions. Under the first condition the devices were placed inside of a shoebox and imaged through a hole in the lid. Under the second condition the devices were imaged in a dimly lit room (~3 lux). The cell phone was placed ~15 cm from the surface of the device and the camera was set to image with an ISO of 800, an exposure of +2, focus set to close-up, and automatic white balancing. A comparison of microscope and cell-phone imaging under the two conditions is shown in Figure 3. Automatic image

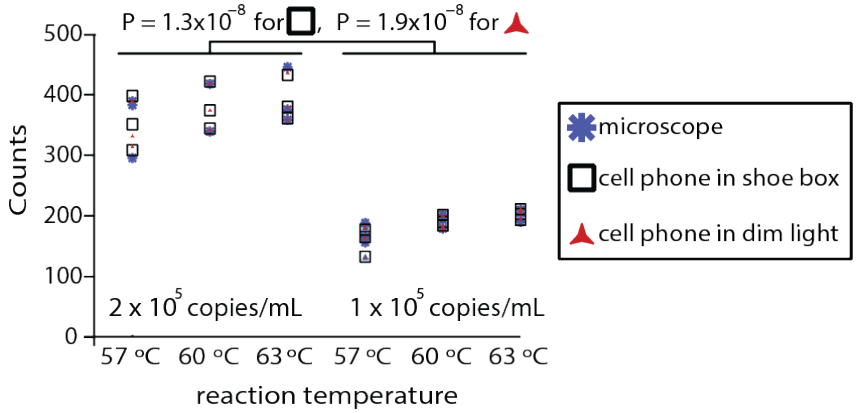

Figure 3: A plot comparing dRT-LAMP experiments imaged with a microscope, a cell phone in a shoe box, and a cell phone in dimly lit room. Cell phone images were manually analyzed using imagej. A statistical difference between the two concentrations remains in both types of cell phone imaging.

processing was tested over a wider 1000-fold dynamic range to ensure that the algorithm was effective over a broad range. The results of the automatic image processing are shown in Figure 4.

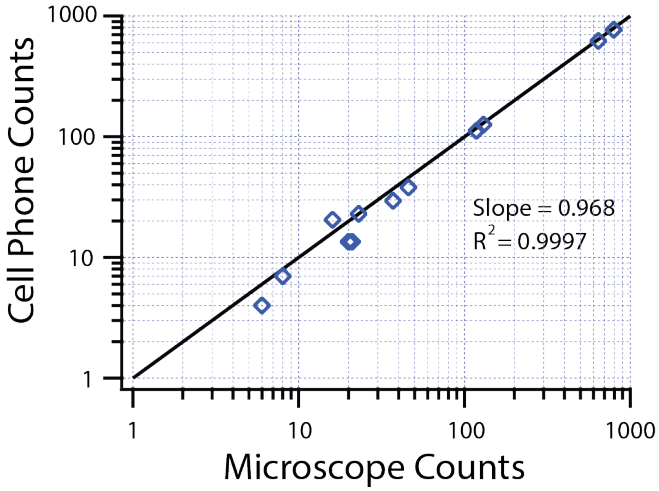

Figure 4: A plot showing results from automatically analyzed images over a 1000-fold concentration change compared with results obtained using the microscope. Excellent correlation is observed between the two sets.

CONCLUSIONS

We demonstrated that a dRT-LAMP assay is more robust to fluctuations in temperature and imaging conditions than a real time RT-LAMP assay. Specifically, we showed that a dRT-LAMP assay was capable of resolving a two-fold concentration change over a variety of different temperature and imaging conditions, whereas a real-time RT-LAMP assay was incapable of doing so. This shows that for LAMP assays, a digital format would be more suitable for LRS conditions where precise environmental controls are unfeasible. We also demonstrated a system whereby the concentration of a dRT-LAMP assay can be automatically determined using an automatic image processing algorithm. This particular system uses remote image analysis, requiring the image to be sent to the cloud before processing can occur. Due to the wide proliferation of cellular networks this should not cause a problem in many locales, as other benefits such as data tracing, archiving, centralized control, and are more easily achievable with this system; however, there are situations where the image processing would be more suitably performed on the cell phone, and the algorithm should easily be able to be modified to work on varying cell-phone platforms.

ACKNOWLEDGEMENTS

This work was supported in part by DARPA Cooperative Agreement No. HR0011-11-2-0006 and by NIH grant No. R01EB012946. This manuscript does not necessarily reflect the position or policy of the U.S. government or these agencies, and no official endorsement should be inferred.

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