CHAPTER 5

FROM THERMAL CYCLING TO ISOTHERMAL: CHALLENGES FOR ROBUSTNESS

5.1 Digital format offers higher robustness than bulk kinetic measurements

Robustness, defined as the consistency of measurement results in the presence of perturbation, is a key factor to consider when designing a POC test. In laboratory settings, robustness is less of a problem because of the availability of dedicated equipment to control experimental conditions precisely. However, such control of reaction conditions often cannot be easily achieved in limited-resource settings, where POC tests are most frequently used. For nucleic acid quantification, nonlinear chemical amplification such as qPCR is usually employed. However, the robustness of such a reaction scheme is not yet fully understood. When quantification is based on the kinetics of an assay, robustness becomes an especially relevant issue, because once the rate of reaction changes, the results of the assay will be influenced directly. While a 3 fold change in HIV viral load could result in a different clinical decision, a change in temperature of a few degrees could potentially shift the results by more than that, as shown below.

We hypothesized that if an end-point digital format instead of real-time kinetic monitoring is used in combination with such exponential amplification, the quantification result will be more robust to potential perturbations, because in a digital format, only identifying yes/no for each compartment is needed for quantification[41a] (Figure 13). In a typical digital analysis, each compartment contains on average less than one molecule, and only those compartments containing target molecules will initiate an amplification reaction and produce a “positive” signal. Therefore, as long as a “positive” or “negative” signal can be identified, knowledge of reaction progress is not required. However, it should be noted that if the initiation of the amplification reaction is influenced by perturbation, the robustness of the digital format needs to be reevaluated.

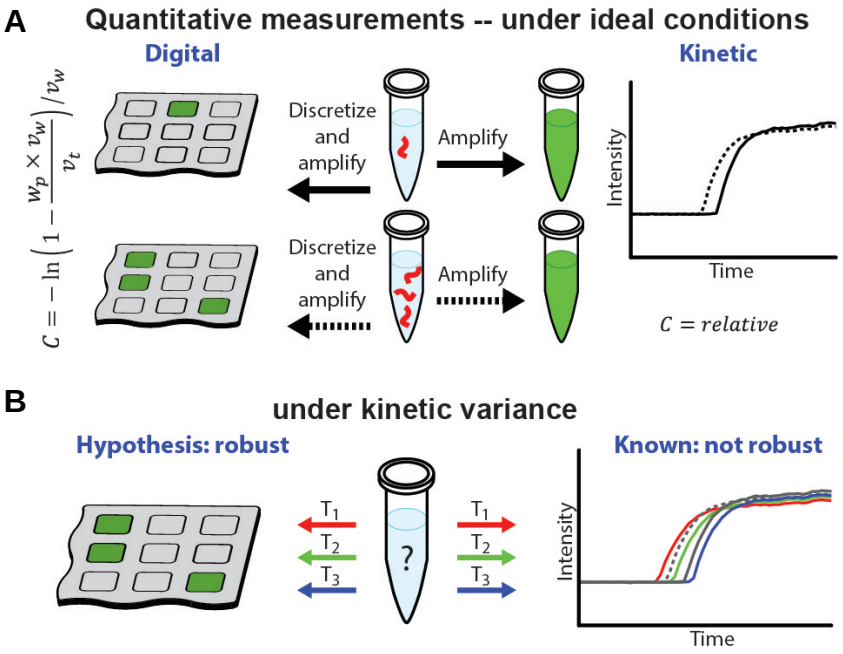

Figure 13 Schematic drawing for the comparison of quantification robustness between digital format and bulk kinetic formats. (A) Quantification of the concentration of the target molecule under ideal conditions: the difference in concentration of the target molecule is reflected by the positive counts in the digital format and the reaction progress in the kinetic measurement. (B) Quantification under non-ideal conditions: the variation in reaction rate does not change the counts in digital format, but influences the reaction progress in the kinetic measurement. Reprinted with permission from reference 41a. Copyright 2013 American Chemical Society.

5.2 Comparison of the robustness between digital quantitative assays and bulk kinetic assays to temperature variation

To evaluate and compare the robustness of digital and kinetic measurements, we used RT-LAMP with an HIV RNA template as a model system. As a rapid, sensitive, and specific isothermal amplification chemistry, RT-LAMP is a good candidate for a POC test, and it has been previously demonstrated on SlipChip microfluidic device for the quantification of HIV viral RNA[42].

We first tested whether quantitative measurements using real-time RT-LAMP assay are robust to temperature fluctuations. Using the two-step protocol developed previously[42] we performed real-time RT-LAMP on an Eco real-time PCR machine. A two-fold change in HIV RNA concentration (1 x 10^5 copies/mL and 2 x 10^5 copies/mL) was successfully distinguished at each

temperature (at 57 °C p = 0.007, at 60 °C p = 0.01, at 63 °C p = 0.04). However, the change in reaction time caused by temperature shifts for each concentration was greater than the change caused by concentration difference (Figure 14A); therefore the two concentrations became indistinguishable if data for different temperatures were grouped together (p=0.25).

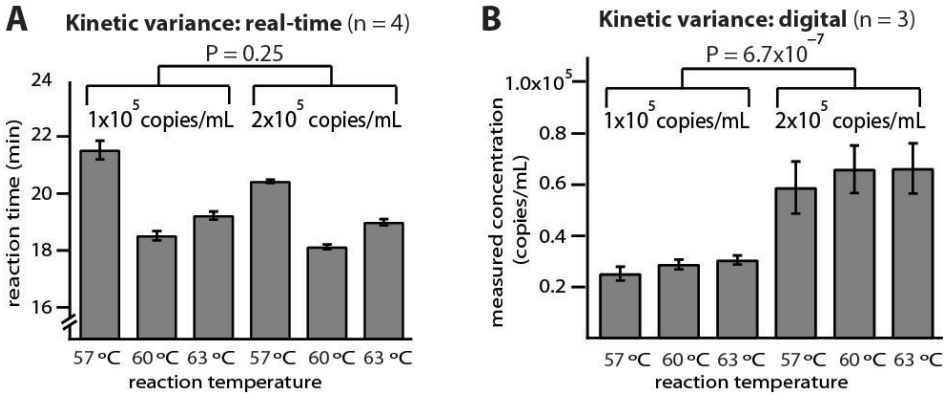

Figure 14 Evaluation of the robustness of real-time RT-LAMP and digital RT-LAMP to variation in temperature. Two concentrations were quantified with A) real-time RT-LAMP and B) digital RT-LAMP assays across a six degree temperature range (n=3). Reprinted with permission from reference 41a. Copyright 2013 American Chemical Society.

The robustness of the digital format of the same RT-LAMP assay was also tested across the same temperature range with the same two concentrations of HIV RNA. The concentration of RNA was determined using Poisson statistics based on the number of positive wells after amplification. Unlike the real-time bulk RT-LAMP assay, the digital RT-LAMP assay could not only distinguish between the two concentrations at each temperature; it could also differentiate the two concentrations despite temperature fluctuations ($p=6.7\times10^{-7}$). We then concluded that the digital RT-LAMP assay for the quantification of HIV RNA is more robust to temperature fluctuation than the real-time RT-LAMP assay.

The lack of robustness of the real-time bulk assay and the robustness of the digital RT-LAMP assay to temperature variation indicated that the amplification rate was influenced significantly, but the probability of reaction initiation was not influenced much by temperature perturbation.

The equilibria of DNA-enzyme, RNA-enzyme, DNA:DNA hybridization, the rate of enzyme attachment and nucleic acid annealing were all temperature-dependent. But again, because the digital format quantification was influenced only by the probability of initiation, it should be more robust to these changes compared with the bulk kinetic assay.

5.3 Comparison of the robustness to changes in assay time for the digital quantitative assay and bulk kinetic assay

Quantification in a digital format is related to the "end-point" of the assay. Ideally, the window for readout should include the minimum number of false negatives (reaction of a single molecule that has not gone to completion) and the minimum number of false positives (non-specific amplification that proceeds at a slower rate), which means the cut-off time should be after all specific amplification turns positive and before non-specific amplification turns positive, and the digital counts should remain stable. To determine the end-point and test whether quantification results of the digital assay were sensitive to this time, we performed a real-time digital RT-LAMP assay with HIV RNA template by imaging the device periodically. Most of the counts turned positive between 20 to 40 minutes, and the change of counts after 40 minutes and before 60 minutes (when false positives started to appear) was less than 10%. A two-fold difference in concentration could be distinguished without any difficulty (Figure 15) regardless of when the reaction was terminated within this 20-minute period.

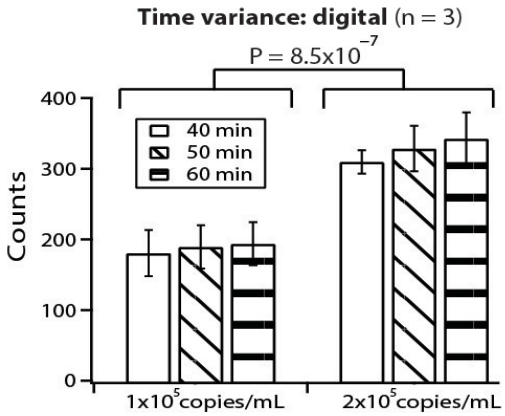

Figure 15 Evaluation of the robustness of digital RT-LAMP to variations in assay time. Two concentrations were quantified with digital RT-LAMP assays and the counts were recorded in a real-time format. Counts at 40, 50 and 60 minutes were plotted (n=3). Reprinted with permission from reference 41a. Copyright 2013 American Chemical Society.

5.4 The integration of digital quantification with cell phone imaging enables direct result readout and feedback

To further evaluate the potential of using digital RT-LAMP for diagnostics in limited-resource settings, we explored the possibility of combining RT-LAMP chemistry with cell phone imaging. We imaged the SlipChip device with the digital pattern after the RT-LAMP reaction with a Nokia 808 Pureview cell phone, which features a 41-megapixel sensor with a pixel size of 1.4 μm. Next, we used a centralized computer with a customized Labview program to process the images, which were automatically uploaded to the server via cloud technology once they were generated (Figure 16). The processed result would then be sent back to the user or doctor for instant feedback. The quantification results of cell phone imaging and data processing were validated against the results obtained by microscope imaging and processed by commercial software.

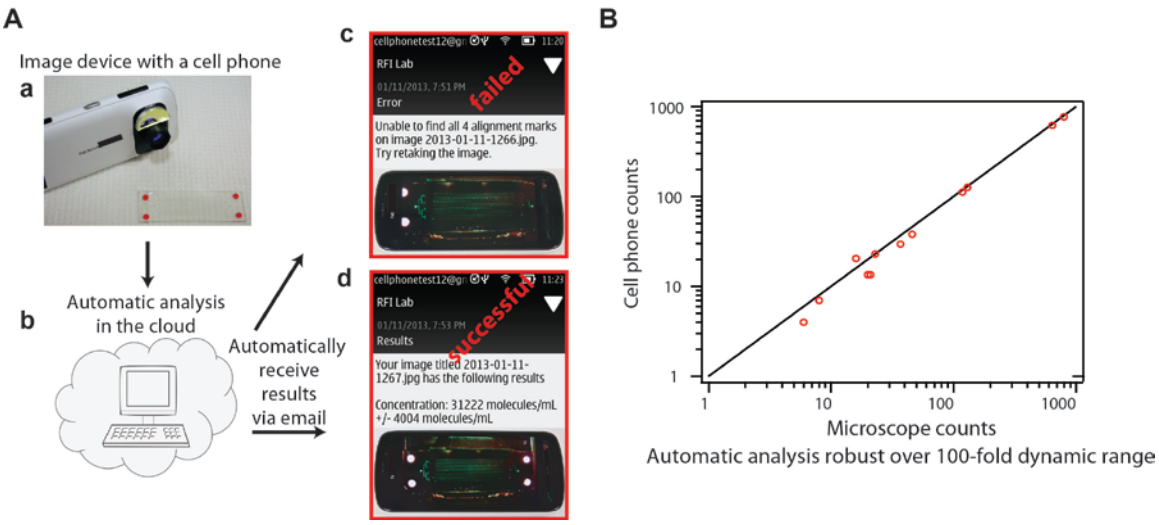

Figure 16 Automatic data analysis workflow and its validation using microscope imaging and Metamorph analysis. A) The image acquisition and analysis workflow with a cell phone and remote server. a)-d): Image was captured by a Nokia phone and transferred via the "cloud" to a remote centralized computer, where software recognized the device and converted the counts into concentration or did not recognize the device and asked the user to retake image. B) Validation of cell phone imaging and analysis with customized Labview program against microscope imaging and analysis with commercial software. Reprinted with permission from reference 41a. Copyright 2013 American Chemical Society.

We understand that the establishment of robust automated counting of HIV RNA molecules using digital amplification chemistry and a cell phone is only part of the full diagnostic assay and several additional advances are needed for it to be used under limited-resource settings, including the integration of a sample preparation module, a simple heating mechanism, and evaluation of robustness to other factors. If all of these elements are addressed, we may see an emergence of rapid amplification schemes that are especially suitable for quantitative measurements under limited-resource settings because of specificity, sensitivity, and robustness.