CHAPTER 4

FROM THERMAL CYCLING TO ISOTHERMAL: CHALLENGES FOR EFFICIENCY

4.1 Eliminating the need for thermal cycling using isothermal amplification

Although digital PCR has been studied and incorporated in various molecular diagnostic assays[16, 25-27], the requirement of thermocycling limits its application in non-laboratory settings. Isothermal nucleic amplification technologies, which use a constant temperature during amplification, reduce the cost and complexity of the instrument, and therefore are more suitable for limited-resource settings. A water bath or very simple chemical heater could be used as the heating source[30]. Many isothermal amplification methods have been invented, including Nucleic Acid Sequence Based Amplification (NASBA) [31], Signal Mediated Amplification of RNA Technology (SMART) [32], Helicase Dependent Amplification (HDA) [33], Loop Mediated Isothermal Amplification (LAMP)[34], Recombinase Polymerase Amplification (RPA) [35], Rolling Circle Replication (RCA) [36], Strand Displacement Amplification (SDA) [37], Exponential Amplification Reaction (EXPAR)[38], Nicking Enzyme Amplification Reaction (NEAR) [39], and DNA circuits[40]. The underlying principles for each amplification chemistry differ, but the key issue for isothermal amplification chemistry is how to generate single-stranded DNA (ssDNA) or ssRNA that allows further annealing of the primer and elongation initiated by the primer to form new strands. This can be achieved by many means, such as with enzyme-assisted double strand opening, strand displacement, and RNA transcription. However, to be applied in a POC test, the reaction should have some other features, including low rates of false positives and false negatives, low requirements for storage conditions, and functionality without complicated instruments for signal readout.

4.2 Loop mediated isothermal amplification (LAMP)

LAMP chemistry is a widely adopted isothermal amplification method that requires constant reaction temperature at 60-65 °C and the participation of only one enzyme (*Bacillus*

stearothermophilus DNA polymerase). LAMP amplifies DNA with high specificity, efficiency and rapidity[34]. It employs a set of four or six specially designed primers that target six or eight distinct regions on the DNA sequence (Figure 8), which guarantees high specificity. The primers are designed in such a way that primer annealing and extension trigger strand displacement, which further produces single-stranded loop structures, allowing further primer annealing and strand displacement (Figure 8). The existence of multiple loop structures on the same amplicon strand significantly increases the number of nucleotides being incorporated per primer annealing event, resulting in the generation of a large number of pyrophosphate as the byproduct of DNA synthesis. Therefore, a calcein-metal based detection chemistry can be used to indicate the accumulation of pyrophosphate during the reaction. This detection chemistry enables the readout of reaction results with a simple image device such as a cell phone or even the naked eye[41]. In summary, LAMP or its derivative RT-LAMP method is a good candidate for the amplification chemistry used in POC molecular diagnostic tests.

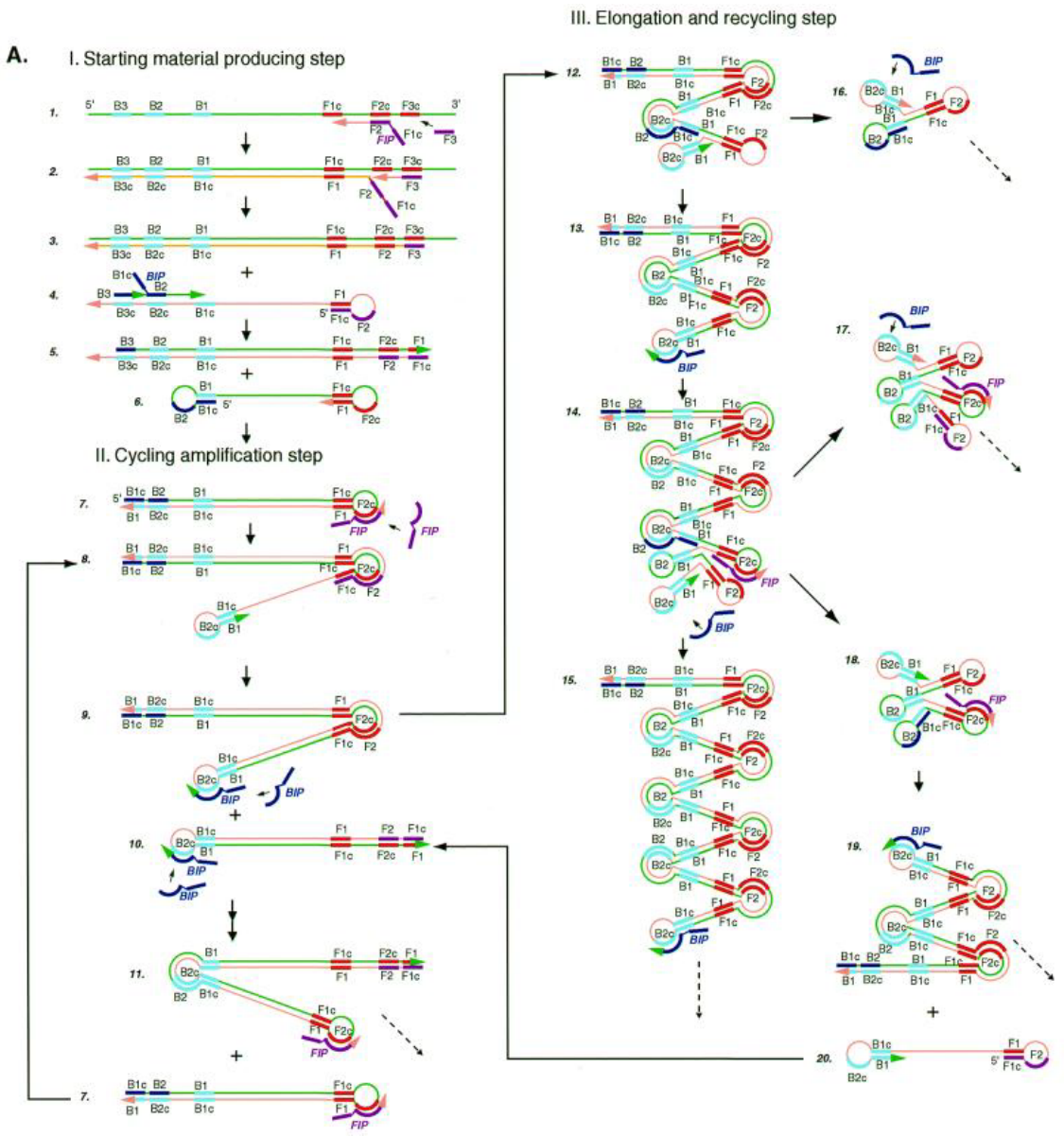

Figure 8 Schematic representation of the mechanism of LAMP. Steps of LAMP reaction are separated into: I) starting material producing step where the double looped structure is generated; II) cycling amplification step and III) elongation and recycling step. Reproduced from reference 34 by permission of Oxford University Press.

4.3 A problem to be carefully evaluated for digital amplification: efficiency

Here we use RT-LAMP for HIV RNA quantification as a model system to answer the question of whether digital RT-LAMP is an attractive alternative to real-time RT-PCR for quantitative diagnosis in limited-resource settings[42]. Although digital LAMP has been demonstrated previously with dsDNA target[43], digital RT-LAMP has never been reported before. The reverse transcription step is necessary when targeting a variety of infectious disease, but could potentially add complexity and introduce issues. We found that performing digital RT-LAMP in a single step on SlipChip is indeed problematic for quantifying HIV viral RNA: the quantification result using digital RT-LAMP was only 2.0% of that achieved using digital RT-PCR with the same template concentration. In this experiment, we compared quantification results of HIV viral RNA at four dilutions using two different digital chemistries—digital RT-PCR and digital RT-LAMP. Digital RT-PCR with primers targeting the LTR region of HIV was used as the standard because it has been validated previously[28]. For RT-LAMP, we used a commercial kit and a set of primers targeting the p24 gene modified based on previous publication[44]. We observed dilution curves with good linearity for both digital chemistries listed above; however the absolute values were dramatically different: the same concentration of RNA gave quantification values that differed by ~50 fold, with digital RT-LAMP giving lower digital counts. We also performed digital RT-PCR with B3/F3 primers taken from the LAMP primer set for comparison and found that even with the same amplification chemistry, the quantified value still differed by 2 fold, with B3/F3 primers giving lower digital counts.

To facilitate the discussion, we defined the percentage of amplified molecules out of the total number of input molecules as “efficiency”. Because digital quantification relies on counting the absolute number of molecules, one essential assumption is that every molecule gets amplified. In other words, people always assume that the efficiency is 100% for digital quantification, which has been shown to be not true in our case. Digital quantification methods with low efficiency,

such as digital RT-LAMP and digital RT-PCR with B3/F3 primers, will not give accurate results and will be inadequate for quantifying lower concentrations of analytes. This is a problem, especially in the case of HIV viral load, where changes most often in the range of 50 to 5,000 copies of RNA per mL need to be quantified to make clinical decisions.

4.4 Improving efficiency by separating sequential reactions from one another

To understand the mechanism leading to this inefficiency, we tested a few hypotheses and devised a way to address the problem to a considerable extent. We hypothesized that the RT-LAMP reaction consists of two stages: initiation before a dumbbell-shaped ssDNA is formed (Figure 8) and propagation after the cycling amplification starts. We hypothesized that the propagation step does not influence the efficiency, but only the time required for the amplification to go to completion; therefore it is the initiation stage that is critical in determining the efficiency. We designed experiments to test which stage determined the efficiency of amplification and validated the hypothesis. Here, the digital format enables us to see this "efficiency" directly reflected as the count of amplification and to separate part of the initiation stage from the other steps. To decouple the reverse transcription step from the amplification step, we designed a SlipChip device to perform RT-LAMP reaction in two steps (Figure 9).

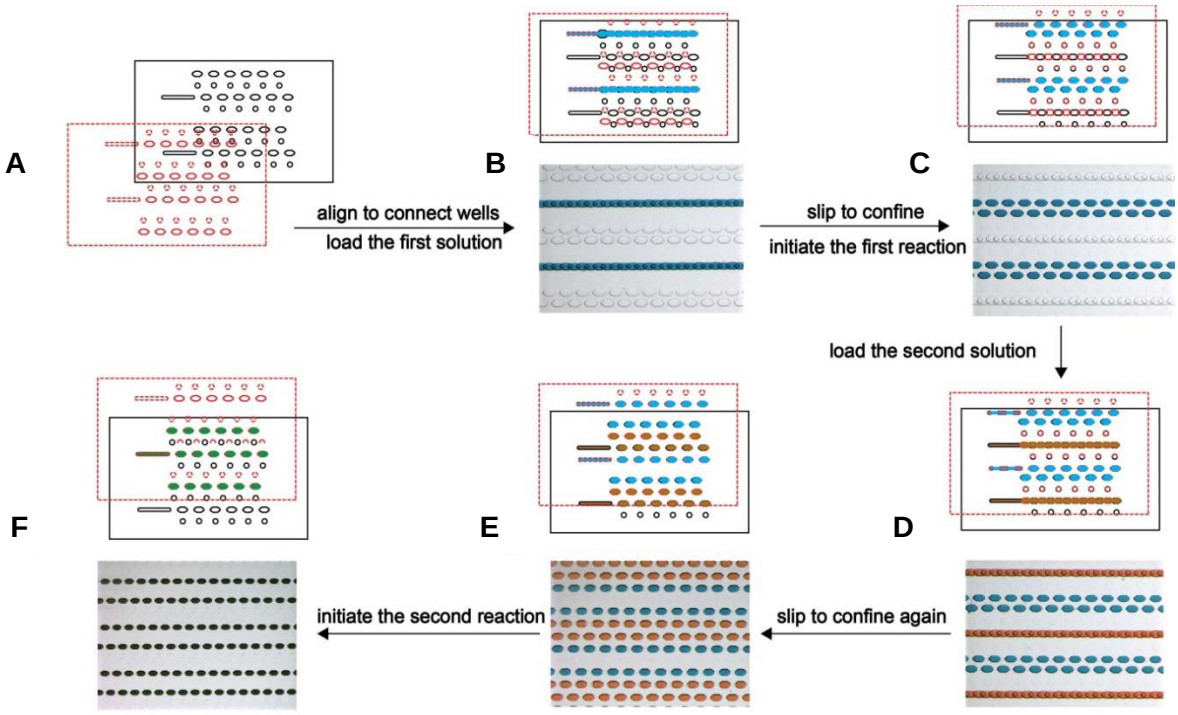

Figure 9 Schematic drawings and bright field images describing the operation of SlipChip for two-step digital experiments. A) Top piece (black) and bottom piece (red) facing each other before assembly. B) The two pieces were aligned to connect ducts and wells. Solution 1 (blue) was introduced along the connected channel. C) Slipping caused the fluid stream to break and isolated droplets were generated and confined in each well. The first step of reaction could be triggered at this stage. D) A second solution (orange) was loaded from another inlet along another set of connected wells and ducts. E) An additional slip generated isolated droplets. F) The two solutions were mixed on the device and the second step of the reaction was triggered simultaneously for all molecules. Reprinted with permission from reference 42. Copyright 2013 American Chemical Society.

With the two-step SlipChip device, we evaluated the influence of several factors in the reverse transcription step, including the orders of primers annealing and the property of different enzymes. We found that when the following changes were made, the efficiency was improved by 10 fold (Figure 10A): 1) only having BIP primer in the first RT step to exclude the interference of other primers; 2) replace AMV reverse transcriptase with Superscript III reverse transcriptase; 3) adding thermostable RNase H in the second step.

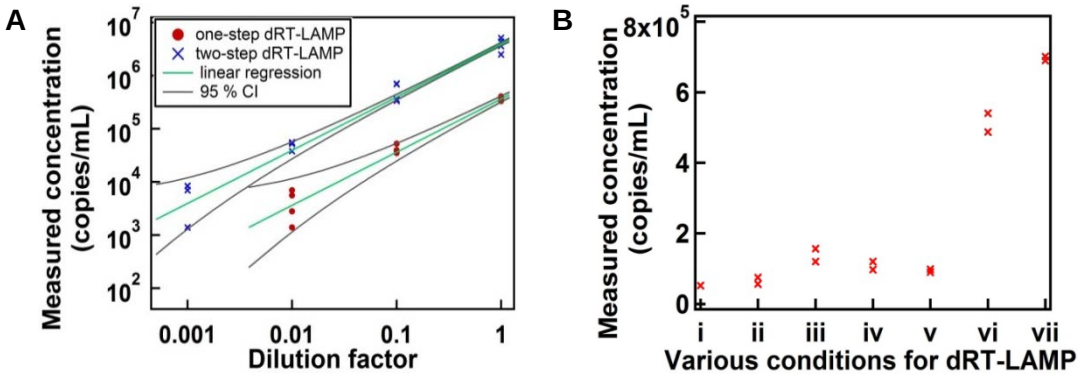

Figure 10 Quantification results of HIV viral RNA concentration (copies/mL) with digital RT-LAMP using different protocols. (A) Quantification results of HIV viral RNA (copies/mL) in a serial dilution experiment on SlipChip using one-step digital RT-LAMP and two-step digital RT-LAMP (n=4). Error bars represent standard deviation. (B) i-vii: one-step digital RT-LAMP; two-step digital RT-LAMP, all primers in RT step, AMV RT; two-step, BIP in RT step, AMV RT; two-step, BIP in RT step, Superscript III; two-step, BIP in RT step, AMV RT, with RNase H; two-step, BIP in RT step, Superscript III, with RNase H; and two-step, BIP in RT step, Superscript III, with RNase H, 0.5x calcein (n=2). Reprinted with permission from reference 42. Copyright 2013 American Chemical Society.

We first hypothesized that if instead of BIP primer, LOOP_B or B3 primer hybridize first to the RNA target and initiate reverse transcription, they would hinder the formation of looped structures on the B side. To test this hypothesis, we tested the efficiency when only BIP primer was present in the first step versus the efficiency when all primers were present, and found a 2 fold higher efficiency for the former. We then evaluated the performance of two different reverse transcriptases to see if the property of the enzyme played a role here. It was found that replacing the AMV reverse transcriptase with Superscript III, which has been reported to have a higher yield, led to no efficiency change. However, when RNase H was added to the second solution, the efficiency improved to 25% (Figure 10B). This could be attributed to the lack of RNase H activity and strand displacement activity in Superscript III—although it synthesized more cDNA, the release of cDNA was prevented.

To exclude the influence of the reverse transcription step, we performed digital LAMP assay with a synthetic 225 nucleotide ssDNA of the same sequence as the HIV RNA as template. The hypothesis behind this was that if the formation of desired cDNA was no longer the bottleneck,

digital LAMP using ssDNA as the template would have the same efficiency as two-step digital RT-LAMP starting from HIV RNA. The experiment results agreed with our expectation: the efficiency was quite similar between digital RT-LAMP for HIV RNA (39%) and digital LAMP for ssDNA (33%) compared to digital RT-PCR with B3/F3 primer, which indicated that the reverse transcription is not the efficiency-limiting step in the two-step protocol. Based on the experimental observation above, we proposed a new mechanism as shown in Figure 11.

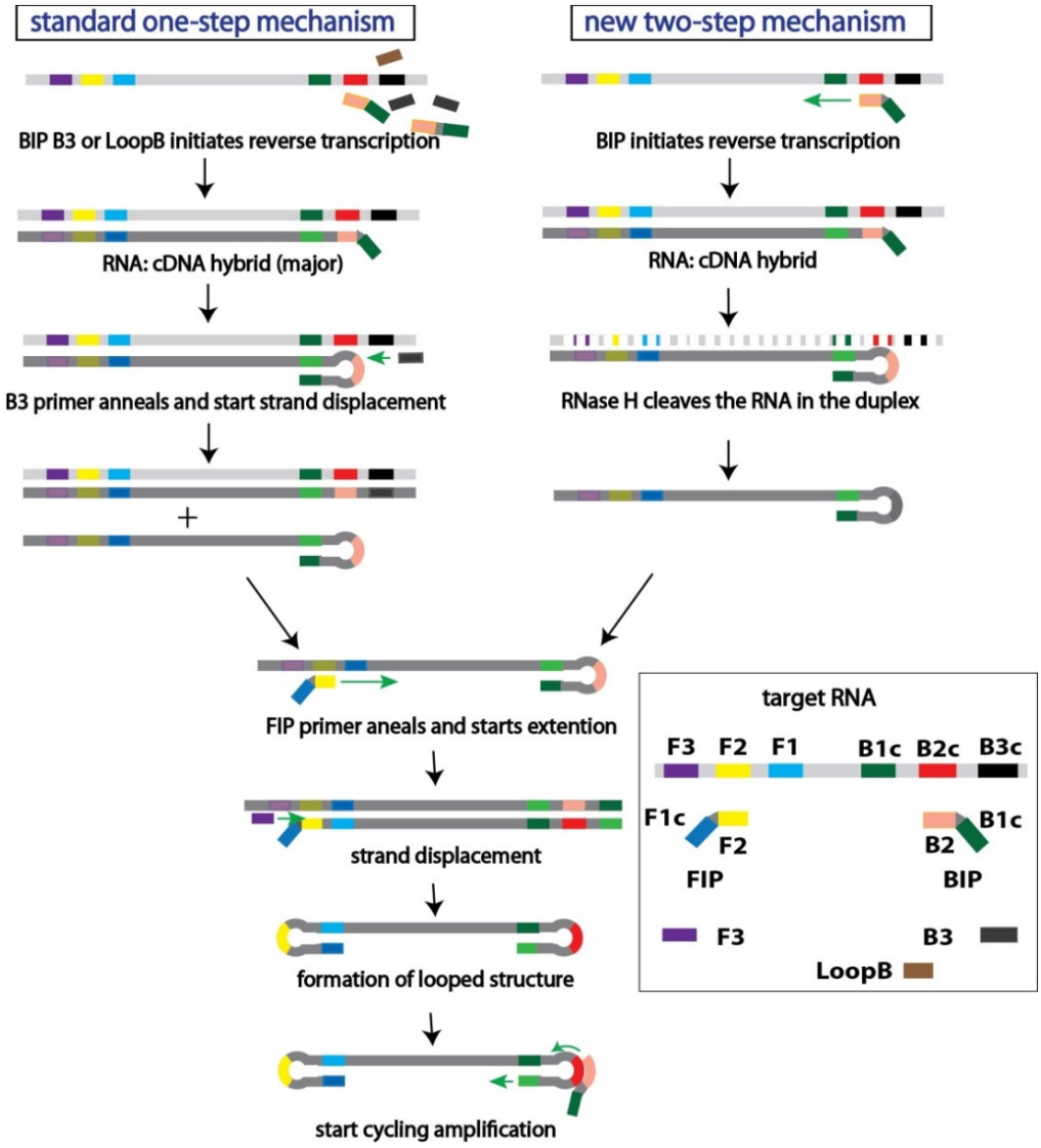

Figure 11 A comparison of the new two-step RT-LAMP mechanism and the published one-step RT-LAMP mechanism. In the initial step, the two-step mechanism uses only BIP primer, while the one-step protocol uses all primers together. After the formation of the DNA:RNA hybrid, the two-step mechanism uses RNase H instead of B3 primer-initiated strand displacement to release the cDNA. The two mechanisms converge after the formation of first-strand cDNA that is looped on one side. Figure truncated; details of cycling amplification including the loop primers' annealing not shown. Inset shows designated primers—B2 and B2c, B3 and B3c, F1 and F1c are complementary sequences, respectively. Reprinted with permission from reference 42. Copyright 2013 American Chemical Society.

4.4 Mismatches between certain primers and templates reduces efficiency

The developed two-step digital RT-LAMP protocol was tested against four patient samples to evaluate the influence of sequence variation to the efficiency of digital RT-LAMP. As shown in Figure 12, the digital RT-LAMP efficiency were 43% ± 9%, 139% ± 33%, 37% ± 20%, and 0.9% ± 0.2% compared to digital RT-PCR with LTR primers for patient sample #1-4. Sequencing results showed that there were 3, 2, 5 and 5 point mutations in the priming regions of sample #1-4, respectively. Therefore, we concluded that the efficiency of digital RT-LAMP is sensitive to mutations in the priming region of the RNA sequence. The efficiency was dramatically increased (the rightmost column compared to the column that is second from the right) from 0.9% ± 0.2% to 22% ± 1 % when mutations in the critical priming position (such as 3' or BIP primer) were corrected. The high specificity of the LAMP reaction often results in relatively low tolerance to mutations and therefore it would be valuable for the assay to maintain both high specificity and good tolerance to mutations. To achieve this goal, more than one set of primers could be included in a single assay to ensure a higher probability of accurate readings, although this idea needs to be tested experimentally.

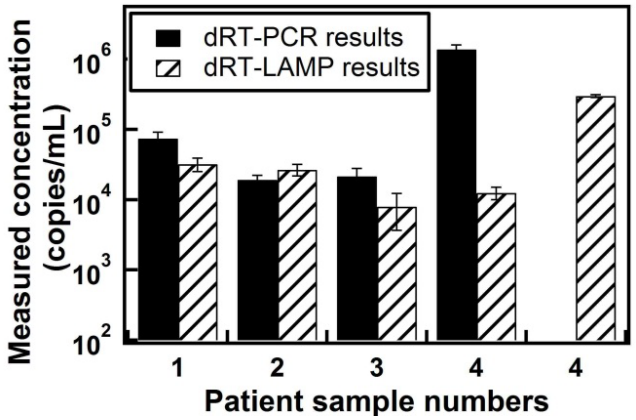

Figure 12 Quantification results of patient HIV RNA samples with digital RT-LAMP and digital RT-PCR. Rightmost column represents the quantification results for sample #4 after primers correction for mutations. Error bars represent standard deviation (n=3). Reprinted with permission from reference 42. Copyright 2013 American Chemical Society.

Single-molecule counting using digital LAMP and digital RT-LAMP is attractive because it is isothermal and does not require thermocycling equipment, and it also provides a bright signal

from the calcein detection system that should be readable by a cell phone. We explored the possibility of using digital RT-LAMP in the quantification of HIV viral load and proposed and tested a two-step protocol for improving the efficiency of this assay. We want to bring to researchers' attention that digital amplification technologies should not always be assumed to provide absolute quantification until validated against a standard.

We have developed new features of the SlipChip microfluidic platform that enable the manipulation of liquid in multiple steps. It not only provide advantages for studying complicated systems, where each step can be isolated and analyzed separately, but also offers possibilities for performing multistep reactions on single targets that need to be isolated, such as single cells. This project also opens the door for performing HIV viral load quantification using isothermal chemistry and simple microfluidic platforms.