

APPENDIX A

Authorship contribution statement and experimental section for Chapter 2

In the following publication¹⁸

Huynh, T.; Sun, B.; Li, L.; Nichols, K. P.; Koyner, J. L.; Ismagilov, R. F., Chemical Analog-to-Digital Signal Conversion Based on Robust Threshold Chemistry and Its Evaluation in the Context of Microfluidics-Based Quantitative Assays. *J. Am. Chem. Soc.* **2013**, *135* (39), 14775-14783.

T.H. and R.F.I. designed the SlipChip experiments.

T.H. was the lead researcher for all figures (Figure 2 and Figure 3 in this dissertation) except for Figure 3 (Figure 4 in this dissertation).

B.S. performed the experiments for Figure 3 (Figure 4 in this dissertation) and helped with FEP coating of the device.

L.L. helped on chip design with the washing channel

K.P.N helped with device surface coating.

J.L.K provided patient samples.

T.H. and R.F.I. wrote the paper.

Experimental section (as described in the publication above)

Chemicals and materials

Phosphate buffer (PB) was a solution of sodium phosphate 0.1 M, pH 7 with Pluronic F127 Prill (BASF) 1 mg/mL. This buffer was made from NaH_2PO_4 (Fisher BP329) and Na_2HPO_4 (Fisher BP332). BAB was a solution of pluronic F127 1 mg/mL in 1xDPBS (10x Gibco 14200 2/2010) pH 7. Starch solution was the suspension of cornmeal in phosphate buffer heated in a boiling water bath for 10 minutes and cooled down to room temperature. The supernatant was then filtered through a syringe filter with a 5- μm membrane to give the starch solution. Inhibitor was a gift by Roman Manetsch and Jordany Maignan (University of South Florida). It was dissolved in DMSO (Fisher D128) to make a 1 mM solution, which was diluted in PB into lower concentrations. Substrate is a mixture of 98 μL starch solution, 1 μL acetylthiocholine (AcSCh) (Sigma A5626) solution (0.4 M in PB), and 1 μL of the 4.016 mL solution of NaI (798.07 mg) (Fisher S324) and I_2 (101.93 M) (Fisher I37) in PB were vortexed in a 600- μL microcentrifuge tube. The substrate mixture was made freshly for each assay. Antibody mixture was 0.709 μL bead-antibody (141 mg/mL), 0.500 μL antibody-biotin (4 OD/cm), and 18.8 μL BAB mixed by pipetting at the time of the assay. Avidin-AChE solution was 1 μL of avidin-AChE (3.36 OD/cm) mixed with 66.2 μL BAB, and stored at 0–4° C.

Conjugation

Bead-antibody: Cystatin C antibody clone 24 (Genway, cat#20-511-242278) was conjugated to tosylated paramagnetic beads (Invitrogen, cat#65501) following the manufacturer's instructions. The approximated final concentration was 141 mg/mL (beads) in PBS (pH 7 with BSA 0.1%, Tween 20 0.05%, and sodium azide 0.02%). The mass of antibody was approximated at 1/8 the mass of beads.

Antibody-biotin: Cystatin C antibody clone 10 (Genway, cat#20-511-242277) was conjugated to biotin using Lightning Link (Novus Biological, cat#704-0015) following the manufacturer's instructions. The buffer was PB, and the absorbance at 280 nm was 4 OD/cm.

Avidin-acetylcholinesterase (avidin-AChE): acetylcholinesterase (AChE) (SigmaAldrich, cat#C2888) (1000 units, 1210 units/mg protein) was conjugated to avidin using Lightning Link (Novus Biological, cat#717-0015) (1 mg avidin). The buffer was sodium phosphate 25 mM, pH 7.2 with Pluronic F127 1 mg/mL. The absorbance at 280 nm was 3.36 OD/cm

Robustness with respect to temperature and detection time

We checked the working ranges of temperature and reaction time of the threshold chemistry using absorbance readout with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)₃ (Sigma-Aldrich D8130) (to react with thiocholine, the product of the enzymatic reaction). In each well in the 96-well plates, acetylcholinesterase (AChE) was incubated with the inhibitor for 30 minutes, followed by the addition of a solution of DNTB and acetylthiocholine (AcSCh) and absorbance measurement at 412 nm by a plate reader (BMG LABTECH, POLARstar Omega). The total volume in each well was 80 μ L, and the final concentrations of AcSCh and DTNB were 0.2 mM and 0.15 mM, respectively. The inhibitor concentrations used in each set of experiments were 13, 16, 21, 27, 36, and 46 nM. We also investigated the robustness when the concentrations of the enzyme and the inhibitor were 10 times smaller. The temperatures were 22, 25, 28, 31, and 34 $^{\circ}$ C.

Threshold chemistry in bulk with visual readout

Solutions of 23 μ L of enzyme and inhibitor (in PB) were incubated in the wells of a 96-well plate at room temperature for 30 minutes. Then 20 μ L of the substrate mixture was added in each of the wells. The image was taken after 39 minutes of reaction using a stereoscope with incident light. The image was subtracted from an image taken after 151 minutes of reaction, when all wells had reacted (as a background subtraction), and then corrected with Photoshop (contrast+95).

Device (SlipChip) fabrication

The SlipChip was fabricated by wet-etching glass plates coated with chromium and photoresist (Telic, soda lime 0.7 mm thick, positive photoresist) with exposed areas from photolithography (mask printed by CadArt), with a twice-concentrated etching mixture (666 mL water, 30 g NH_4F , 78 mL HNO_3 70%, 56 mL HF mix (SigmaAldrich 01066)). To etch features of different depths on the same chips, features with shallower depths were covered with packing tape at the beginning and the tape was later removed to expose the features during the process. The depths of the features measured by a contact profiler (Dektak 150) were 142 μm for the analyte wells of the top plate, 22 μm for the rest of the top plate, and 130 μm for the bottom plate. The inlets (on the top plate) and the alignment holes (on both plates) were formed by drilling the chips using 0.035 in drill bits (Harvey Tool 74335-C4).

Treatment of chip surface

The chips were coated with FEP to prevent wetting of the surface by aqueous solutions in areas that do not contain any features (wells or ducts). The glass chips were cleaned in H_2SO_4 98% : H_2O_2 30% (3:1 v/v) for 1 hour, then sonicated in NaOH (concentrated, ~ 50 g/L). They were then dip-coated in FEP emulsion (Fuel Cell Earth LLC, cat#TE9568-250) diluted 3 times with Millipore water (advancing and withdrawing speeds of 10.8 and 1.8 cm/min, respectively). The coated chips were baked on a hot plate at 250 °C (30 min total time, including heating up from room temperature and baking at 250 °C) and at 340 °C for 1 minute, then cooled in air at room temperature. The chips were then incubated in deionized water for 2.5 hours, and then dried with a N_2 stream. The features designed to contain aqueous droplets had their FEP layer removed either completely (in the case of the top plate) or partially (in the case of the bottom plate) to ensure retention of the droplets during droplet deposition and transfer during the assay. The FEP layer in wells and washing channels on the bottom plate were partially removed by laser

machining (Resonetics RapidX250 system, with demagnification of 10, constant energy mode of 50 mJ with 50 % attenuator, 75-mm lens, and fluence of 9.32 J/cm²). The ablated areas were approximately half of those of the features and were centered in the features. The FEP coating of wells on the top plate were removed manually and completely with a needle (27G1/2, Beckton-Dickinson 305109) under deionized water. The process was performed under a stereoscope. Another purpose of the removal of FEP in the analyte wells was so that the analyte solutions would wet and fill the wells completely.

Setting up the assay

The bottom plate was put in a square petri dish (Fisher 08-757-11A) filled with FC-40 (3M). The side with features was facing up, and the plate was separated from the bottom of the petridish by the ends of insect pins inserted into the alignment holes. Washing solutions were deposited into the washing channels by pipetting with volumes of 0.5 μ L (BAB), 1.2 μ L (BAB), and 0.7 μ L (PB), following the progress of the assay. Solutions (1 μ L) of inhibitor were deposited into inhibitor wells by pipetting with low-retention pipette tips. Solutions of antibody mixture and substrate mixture were made at the time of the experiment. Plugs were made and deposited onto the bottom piece.⁴ Plugs were made with a cross channel (Idex Health & Science P-634). FC-40 flowed into two opposite inlets with rate of 80 μ L/min each (B-D plastic syringe 1 mL (B-D REF 309602), needle 23G1½, B-D 305194, tubing FEP 1/16x0.20x50ft (Idex Health & Science 1548L)). The aqueous solution flowed into the remaining inlet at 40 μ L/min (B-D plastic syringe 1 mL (B-D REF 309602), needle 18G1½, B-D 305196, tubing FEP 1/16x0.20x50ft (Idex Health & Science 1548L)). The syringe for the aqueous inlet was filled with FC-40, then with a small air bubble, then the actual aqueous solution so that the aqueous solution stayed in the FEP tubing only. The pumps used to make plugs were Harvard PHD 2000. Plugs were put into FEP tubing 1/16x0.20x50ft (Idex Health & Science 1548L). The tip of the outlet tubing was cleaned using a piece of Kimwipe (Kimberly-Clark) wetted with D.I. water prior to making each set of plugs.

After the series of plugs were formed in the outlet tubing, the flow rate was slowed down to 20 $\mu\text{L}/\text{min}$ during deposition, and to 2 $\mu\text{L}/\text{min}$ during the movement to another bottom plate. The sequence of solutions that were deposited was as follows: substrate mixture, avidin-AChE, and antibody mixture. The plug-making apparatus was washed by making plugs of BAB between uses with different solutions. After all the solutions were loaded onto the bottom plate, the top plate was placed onto the bottom plate with its alignment holes going over the insect pins. The chips were then taken out of the petri dish with a pair of tweezers, clamped with plastic clothespins (3 on each of two opposite sides of the chip), and aligned with better accuracy under a stereoscope.

Running the assay

Loading: The sample (10 μL) was loaded into the inlet with a pipette tip.

Incubation: When the analyte wells were completely filled with the sample, they were slipped to overlap with the wells containing the antibody mixture. The magnet (long enough to cover the whole row of wells) was used to pull the beads up to the top plate, then to the bottom plate, for a total of 2–3 iterations of back-and-forth movement in the direction of slipping, and the magnet was pulled away from the chip when it was at the middle of the row. The 6 clothespins were replaced with 2 clamps (plastic clamp grips for paper, cut into pieces that fit the chip) on opposite sides of the chip. The chip was incubated in an inverted position (bottom plate up) in a petri dish containing FC-40 at room temperature for 1 hour.

End of incubation: After the incubation, the chip was taken out of the petri dish and held by hand in the proper position (top plate up). The magnet was used to pull the beads to the top plate. The magnet was then held on the top plate during slipping to move the wells containing the beads in contact with the first washing channel.

Washing: The magnet was used to move the beads through the washing phase. The magnet was then held on the top plate near the beads during slipping to move the beads to the next step. The

beads underwent incubation and washing as described above. Each of the incubations with avidin-AChE and the inhibitor also lasted 1 hour at room temperature with the chip in the inverted position in the petri dish containing FC-40.

Initiation of reaction: After the incubation with the inhibitor, the chips were taken out of the petri dish, held by hand in the proper position. The magnet was used to bring the beads into the dumbbell-shaped wells in the top plate. The magnet was held onto the top plate during slipping to bring the wells that contained the beads into contact with the substrate wells. The magnet was used to move the beads to the direction of slipping, then immediately opposite. This moment was set as 0, the beginning of reaction time. During the reaction, the chip was left in air at room temperature. Images of assay results were taken at 70 minutes after the initiation of the reactions.

Handling of images of assay results

Images taken with a digital SLR camera: We used a Canon RebelXT with EFS18-55mm lens. The images were cropped and processed in Photoshop (selective color: hue 0, saturation -100, lightness +50; brightness -5, contrast +50). Images taken with a camera phone: We used an iPhone4 (Apple Inc.). The images were cropped and processed in Photoshop (brightness +50, contrast +50).

Estimation of threshold concentration from assay results of standards and patient samples

The smallest increase of [cystatin C] that led to a change in the position of ON/OFF transition was 0.64–0.75 mg/L (1.2 fold). Therefore, we estimated the threshold concentration in column 2 as $0.69 = (0.64 \cdot 0.75)^{0.5}$ and used this value to estimate the threshold concentrations in other columns, based on 2-fold steps in inhibitor concentrations.

APPENDIX B

Authorship contribution statement and experimental section for Chapter 3

In the following publication

Shen, F.; Sun, B.; Kreutz, J. E.; Davydova, E. K.; Du, W.; Reddy, P. L.; Joseph, L. J.; Ismagilov, R. F., Multiplexed Quantification of Nucleic Acids with Large Dynamic Range Using Multivolume Digital RT-PCR on a Rotational SlipChip Tested with HIV and Hepatitis C Viral Load. *J. Am. Chem. Soc.* **2011**, *133* (44), 17705-17712.

F.S., B.S., J.E.K., W.D. and R.F.I. designed the SlipChip experiments.

F.S. was involved in design and discussion of all figures and was the lead researcher for Figure 1 (Figure 6A in this dissertation), 2 (Figure 6B in this dissertation), 3, 4, and 6.

B.S. was the lead researcher for Figure 5 (Figure 7 in this dissertation).

F.S. and B.S. performed experiments and J.E.K., F.S., B.S. performed data analysis. E.K.D. prepared control RNA and viral RNA used in the experiments.

F.S., B.S., J.E.K. and R.F.I. wrote the paper.

P.L.R and L.J.J. provided the deidentified patient samples, and performed viral load tests with Roche system in parallel.

P.L.R. and L.J.J. also provided suggestions on HIV viral load protocols.

Experimental section (as described in the publication above)

Chemicals and Materials

All solvents and salts obtained from commercial sources were used as received unless otherwise stated. SsoFast EvaGreen SuperMix (2X) was purchased from Bio-Rad Laboratories (Hercules, CA). One-Step SuperScript® III Reverse Transcriptase, iPrep™ purification instrument, and iPrep™ PureLink™ virus kit were purchased from Invitrogen Corporation (Carlsbad, CA). All primers were purchased from Integrated DNA Technologies (Coralville, IA). Bovine serum albumin (20 mg/mL) was ordered from Roche Diagnostics (Indianapolis, IN). Mineral oil, tetradecane, and DEPC-treated nuclease-free water were purchased from Fisher Scientific (Hanover Park, IL). Dichlorodimethylsilane was ordered from Sigma-Aldrich (St. Louis, MO). PCR Mastercycler and in situ adapter were purchased from Eppendorf (Hamburg, Germany). Spectrum food color was purchased from August Thomsen Corp (Glen Cove, NY). Soda-lime glass plates coated with layers of chromium and photoresist were ordered from Telic Company (Valencia, CA). Photomasks were designed using AutoCAD (San Rafael, CA) and ordered from CAD/Art Services, Inc. (Bandon, OR). Microposit™ MFTM-CD-26 developer was purchased from Rohm and Hass Electronic Materials LLC (Marlborough, MA). Amorphous diamond coated drill bits were purchased from Harvey Tool (0.030 inch cutter diameter, Rowley, MA). Adhesive PDMS film (0.063 inch thick) was purchased from McMaster (Atlanta, GA). The MinElute PCR purification kit and QIAamp Viral RNA mini kit were purchased from Qiagen Inc. (Valencia, CA). The OptiQuant®-S HCV RNA quantification panel was purchased from AcroMetrix (Benicia, CA).

Fabrication of SlipChip for multivolume digital RT-PCR

The procedure for fabricating the SlipChip from soda lime glass was based on the procedure described in previous work. To fabricate SlipChip for multivolume digital RT-PCR, wells of two

different depths were etched using a two-step exposing-etching protocol. The soda lime glass plate pre-coated with chromium and photoresist was first aligned with a photomask containing the design for part of the wells. The glass plate was then exposed to UV light using standard exposure protocols. After exposure, the glass plate was detached from the photomask and immersed in developer to immediately remove the photoresist that was exposed to UV light. The underlying chromium layer that was exposed was removed by applying a chromium etchant (a solution of 0.6:0.365 mol/L HClO_4 / $(\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6$). The glass plate was thoroughly rinsed with water and dried with nitrogen gas. The glass plate was then aligned with a second photomask containing the designs of the rest of the wells. The glass plate was then exposed to UV light a second time. After the second exposure, the photomask was detached from the glass plate, and the back side of the glass plate was protected with PVC sealing tape. The taped glass plate was then immersed in a glass etching solution (1:0.5:0.75 mol/L $\text{HF}/\text{NH}_4\text{F}/\text{HNO}_3$) to etch the glass surface where chromium coating was removed in the previous step (areas containing wells of 25 nL, 125 nL, and 625 nL), and the etching depth was measured by a profilometer. After the larger features were etched to a depth of 70 μm , the glass plate was placed in the developer again to remove the previously exposed photoresist in areas containing the patterns for the smaller features. The underlying chromium layer was removed by using the chromium etchant as describe above, and a second glass etching step was performed to etch all features to a further depth of 30 μm . The final SlipChip contained wells of depths of 100 μm and 30 μm was fabricated. After the two-step etching, the glass plate was thoroughly rinsed with Millipore water and ethanol and then dried with nitrogen gas. The glass plate was oxidized using a plasma cleaner and immediately placed in a desiccator with dichlorodimethylsilane for gas-phase silanization. After one hour, the silanized glass plate was thoroughly rinsed with chloroform, acetone, and ethanol, and then dried with nitrogen gas. To re-use the glass SlipChips, each SlipChip was thoroughly cleaned with piranha acid (3:1 sulfuric acid: hydrogen peroxide), then oxidized using a plasma cleaner and silanized as described above.

Assembling the SlipChip

The SlipChip was assembled under de-gassed oil (mineral oil: tetradecane 1:4 v/v). The bottom plate was immersed into the oil phase with the patterned wells facing up, and the top plate was then immersed into the oil phase and placed on top of the bottom plate with the patterned side facing down. The two plates were aligned under a stereoscope (Leica, Germany) and stabilized using binder clips.

Loading the SlipChip

A through-hole was drilled in the center of the top plate to serve as the solution inlet. The reagent solution was loaded through the inlet by pipetting. For the multiplexed design, five through-holes were drilled at the top left corner of the top plate to serve as fluid inlets. For multiplex experiments, five different reaction solutions were placed in the inlet reservoirs, and a dead-end filling adapter was placed on top of the SlipChip to cover all the inlets. A pressure of 18 mmHg was applied to load all the solutions simultaneously. The principle and detailed method for dead-end filling are described in a previous work. Reservoirs were removed after the solution was loaded.

Synthesis and purification of control RNA (906nt)

The control RNA (906 nucleotide) was synthesized from the LITMUS 28iMal Control Plasmid using a HiScribe™ T7 In Vitro Transcription Kit with the manufacture's recommended procedures (New England Biolabs, Ipswich, MA) and purified using MinElute PCR purification kit with manufacture recommended protocols.

Automatic Viral RNA purification from plasma sample

Plasma samples containing the HIV virus were obtained from deidentified patients at the University of Chicago Hospital. Plasma containing a modified HCV virus as a control (25 million IU/mL, part of OptiQuant-S HCV Quantification Panel) was purchased from AcroMetrix (Benicia, CA). A plasma sample of 400 μ L was mixed with 400 μ L lysis buffer (Invitrogen Corporation, Carlsbad, CA) to lyse the virus. Then 2 μ L of control RNA (906 nt) was added to characterize the purification efficiency and concentrating factor. The mixed sample was then transferred into the iPrep™ PureLink™ virus cartridge. The cartridge was placed in the iPrep™ purification instrument and the purification protocol was performed according to the manufacturer's instructions. The final elution volume was 50 μ L, therefore a theoretical eight-fold concentrating factor was expected. The initial concentration of control RNA and the concentration of control RNA in the purified sample after preparation were characterized on the SlipChip. The final concentrating factor was 4.5 for HCV and 6.6 for HIV in the multiplex RT-PCR amplification.

Primer sequences for RT-PCR amplification

Primers for the control RNA (906 nt) were: GAA GAG TTG GCG AAA GAT CCA CG and CGA GCT CGA ATT AGT CTG CGC. The control RNA template was serially diluted in 1 mg/mL BSA solution. The RT-PCR mix contained the following: 30 μ L of 2 \times EvaGreen SuperMix, 1 μ L of each primer (10 μ mol/L), 3 μ L of BSA solution (20 mg/mL), 1.5 μ L of SuperScript® III Reverse Transcriptase, 17.5 μ L of nuclease-free water, and 6 μ L of template solution.

Primer sequences for HIV viral RNA: GRA ACC CAC TGC TTA ASS CTC AA; GAG GGA TCT CTA GNY ACC AGA GT.

Primer sequences for control HCV viral RNA were selected from a previous publication: GAG TAG TGT TGG GTC GCG AA; GTG CAC GGT CTA CGA GAC CTC.

RT-PCR amplification on the SlipChip

To amplify HIV viral RNA, the RT-PCR mix contained the following: 15 μL of $2 \times$ EvaGreen SuperMix, 0.6 μL of each primer (10 $\mu\text{mol/L}$), 1.5 μL of BSA solution (20 mg/mL), 0.75 μL of SuperScript® III Reverse Transcriptase, 10.05 μL of nuclease-free water, and 1.5 μL of template solution. The template solution used here was diluted 250-fold from the original HIV viral RNA stock solution purified from Patient sample 2 using 1 mg/mL BSA solution.

To amplify control HCV viral RNA, the RT-PCR mix contained the following: 15 μL of $2 \times$ EvaGreen SuperMix, 0.25 μL of each primer (10 $\mu\text{mol/L}$), 1.5 μL of BSA solution (20 mg/mL), 0.75 μL of SuperScript® III Reverse Transcriptase, 10.25 μL of nuclease-free water, and 2 μL of template solution. The template solution was diluted 5-fold from the original control HCV viral RNA stock solution purified from OptiQuant-S HCV Quantification Panel.

To amplify the control RNA (906 nt) in the HIV sample, the RT-PCR mix contained the following: 20 μL of $2 \times$ EvaGreen SuperMix, 1 μL of each primer (10 $\mu\text{mol/L}$), 2 μL of BSA solution (20 mg/mL), 1 μL of SuperScript® III Reverse Transcriptase, 13 μL of nuclease-free water, and 2 μL of HIV viral RNA stock solution after sample preparation.

The concentration of control RNA (906 nt) before sample preparation was characterized with the RT-PCR mix contained the following: 20 μL of $2 \times$ EvaGreen SuperMix, 1 μL of each primer (10 $\mu\text{mol/L}$), 2 μL of BSA solution (20 mg/mL), 1 μL of SuperScript® III Reverse Transcriptase, 13 μL of nuclease-free water, and 2 μL of template solution. The template was prepared by diluting 2 μL of stock control RNA (906nt) solution into 400 μL of 1 mg/mL BSA solution.

The amplifications were performed using a PCR mastercycler machine (Eppendorf). To amplify the RNA, an initial 30 min at 50 °C was applied for reverse transcription, then 2 min at 95 °C for enzyme activation, followed by 35 cycles of 1 min at 95 °C, 30 sec at 55 °C and 45 sec at 72 °C.

After the final cycle, a final elongation step was applied for 5 min at 72 °C. This thermal cycling program was applied to all experiments.

Image acquisition and analysis

Bright-field images were acquired using a Canon EOS Rebel XS digital SLR camera (Lake Success, NY). Other bright-field images were acquired using a Leica stereoscope. All fluorescence images were acquired by Leica DMI 6000 B epi-fluorescence microscope with a 5X / 0.15 NA objective and L5 filter at room temperature. All fluorescence images were corrected for background by using an image acquired with a standard fluorescent control slide. All the images were then stitched together using MetaMorph software (Molecular Devices, Sunnyvale, CA).

APPENDIX C

Authorship contribution statement and experimental section for Chapter 4

In the following publication

Sun, B.; Shen, F.; McCalla, S. E.; Kreutz, J. E.; Karymov, M. A.; Ismagilov, R. F., Mechanistic Evaluation of the Pros and Cons of Digital RT-LAMP for HIV-1 Viral Load Quantification on a Microfluidic Device and Improved Efficiency via a Two-Step Digital Protocol. *Anal. Chem.* **2013**, *85* (3), 1540-1546.

B.S. and R.F.I. designed the SlipChip experiments.

B.S. performed experiments and data analysis and was the lead researcher for all figures (Figure 9 to Figure 12 in this dissertation).

F.S. performed exploratory experiments for primer optimization.

S.E.M. performed ssDNA synthesis.

B.S., S.E.M., and R.F.I. wrote the paper.

J.E.K. contributed to the design of the two-step SlipChip and data analysis.

M.A.K. provided suggestions on experiment design and assisted the data acquisition for Figure 2 (Figure 9 in this dissertation).

Experimental section (as described in the publication above)

Chemicals and materials

All solvents and salts purchased from commercial sources were used as received unless otherwise stated. The LoopAmp® DNA amplification kit, the LoopAmp® RNA amplification kit, and the Calcein fluorescence indicator kit were purchased from SA Scientific (San Antonio, TX, USA). SsoFast EvaGreen Supermix (2X) was purchased from Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin (BSA) and λ -DNA isolated from bacteriophage λ cl857 Sam 7 were purchased from Roche Diagnostics (Indianapolis, IN). Hybridase™ Thermostable RNase H was purchased from Epicentre Biotechnologies (Madison, WI). All primers were ordered from Integrated DNA Technologies (Coralville, IA). Mineral oil (D Nase, R Nase, and Protease free), tetradecane, and DEPC-treated nuclease-free water were purchased from Fisher Scientific (Hanover Park, IL). Dichlorodimethylsilane was purchased from Sigma-Aldrich (St. Louis, MO). AcroMetrix® HIV-1 Panel Copies/ml, EXPRESS One-Step SYBR® GreenER™ Universal, iPrep™ purification instrument, and iPrep™ PureLink™ virus kit were purchased from Life Technologies (Grand Island, NY). PCR Mastercycler and in situ adapter were purchased from Eppendorf (Hamburg, Germany). Spectrum food color was purchased from August Thomsen Corp (Glen Cove, NY). Photomasks were designed in AutoCAD 2012 and ordered from CAD/Art Services, Inc. (Bandon, OR). Soda-lime glass plates coated with layers of chromium and photoresist were ordered from Telic Company (Valencia, CA). Polycarbonate SlipChip devices were designed in AutoCAD 2012 and purchased from microfluidic ChipShop GmbH (Jena, Germany).

Fabrication of SlipChips

Two-step exposing-etching protocol was adapted to create wells of two different depths (5 μm for thermal expansion wells, 55 μm for all the other wells). After etching, the glass plates were

thoroughly cleaned with piranha acid and DI water, and dried with nitrogen gas. The glass plates were then oxidized in a plasma cleaner for 10 minutes and immediately transferred into a desiccator for 1 hour of silanization. They were rinsed thoroughly with chloroform, acetone, and ethanol, and dried with nitrogen gas before use.

Plastic polycarbonate SlipChip devices were directly oxidized in a plasma cleaner for 15 minutes after they were received from microfluidic ChipShop GmbH, and then transferred into a desiccator for 90 minutes of silanization. They were soaked in tetradecane for 15 minutes at 65 °C and then rinsed thoroughly with ethanol, then dried with nitrogen gas before use. Plastic SlipChip devices were not reused.

Assembling and loading the SlipChips

The SlipChips were assembled under de-gassed oil (mineral oil: tetradecane 1:4 v/v). Both top and bottom plates were immersed into the oil phase and placed face to face. The two plates were aligned under a stereoscope (Leica, Germany) and fixed using binder clips. Two through-holes were drilled in the top plate to serve as fluid inlets. The reagent solution was loaded through the inlet by pipetting.

HIV viral RNA purification from AcroMetrix® HIV-1 Panel Copies/mL

400 µL plasma containing a modified HIV virus (5 million copies/mL, part of AcroMetrix® HIV-1 Panel Copies/mL) was loaded onto the iPrep™ PureLink® Virus cartridge. The cartridge was placed in the iPrep™ purification instrument and the purification protocol was performed according to the manufacturer's instructions. The elution volume was 50 µL. The purified HIV viral RNA was diluted 10, 102, 103 fold in 1 mg/mL BSA solution, aliquoted and stored at -80 °C for further use. HIV viral RNA purified from patient plasma was also aliquoted and stored at -80 °C upon receipt.

ssDNA synthesis

HIV cDNA was created by reverse transcription of the purified AcroMetrix® HIV RNA using the SuperScript III First-Strand Synthesis SuperMix according to the manufacturer's instructions. Briefly, a mixture of purified HIV RNA (10-fold diluted from the direct elution), 100 nM B3 primer, 1x Annealing buffer, and water were heated to 65 °C for 5 minutes and then placed on ice for 1 minute. A reaction mix and SuperScript III/RNase Out enzyme mix were added to the reaction for a final volume of 40 µL, and the mixture was placed at 50 °C for 50 minutes. The mixture was then heated to 85 °C for 5 minutes to deactivate the reverse transcriptase, chilled on ice, split into 5 µL aliquots, and frozen at -20 °C until further use. Biotin-labeled DNA was created in a PCR reaction containing a 1:50 dilution of the HIV cDNA, 500 nM biotin-B3 and F3 primers, 500 µM dNTPs, 1 U/µL Phusion DNA polymerase and 1x of the associated HF buffer mix. After an initial 1 minute enzyme activation step at 98 °C, the reaction was cycled 39 times at 98 °C for 10 s, 58 °C for 15 s, and 72 °C for 15 s, and finished with a 5 minute polishing step at 72 °C. The resulting DNA product was run on a 1.2% agarose gel in TBE buffer stained with 0.5 µg/mL ethidium bromide. The specific band was cut out and purified using the Wizard SV gel and PCR cleanup kit according the manufacturer's instructions and eluted into 50 µL of nuclease-free water. 50 µL of streptavidin MyOne T1 magnetic beads were primed by slow-tilt rotation for 24 hours in 20 mM NaOH¹⁴. The beads were washed 1 time with water and 4 times with binding buffer (5 mM Tris, 0.5 mM EDTA, 1 M NaCl, 0.05% Tween-20) and re-suspended in 30 µL of 2x concentrated binding buffer. 30 µL of PCR product was added to the beads and incubated for 15 minutes while gently rotating to allow binding of the DNA to the magnetic beads. The beads were separated with a magnet, the supernatant was removed, and the beads were re-suspended in 40 µL of 20 mM NaOH and incubated for 10 minutes on a rotator to separate the non-biotinylated strand. The beads were then separated with a magnet, and the supernatant containing the ssDNA was collected and mixed with 20 µL of 40 mM HCl. The resulting ssDNA was then purified

using an ssDNA/RNA cleaner and concentrator kit, eluted in 20 μ L water, and run on an Agilent RNA nano bioanalyzer to confirm the size and integrity of the final product.

Primers used for RT-LAMP amplification and RT-PCR amplification

F3	ATTATCAGAAGGAGCCACC
B3	CATCCTATTTGTTTCCTGAAGG
FIP	CAGCTTCCTCATTGATGGTCTCTTTTAACACCCATGCTAAACACAGT
BIP	TATTGCACCAGGCCAGATGATTTTGTACTAGTAGTTCCTGCTATG
LOOPF	TTTAACATTTGCATGGCTGCTTGAT
LOOPB	GAGAACCAAGGGGAAGTGA

PCR_Forward: GRA ACC CAC TGC TTA ASS CTC AA

PCR_Reverse: GAG GGA TCT CTA GNY ACC AGA GT

dRT-LAMP and dLAMP amplification on SlipChip

To amplify HIV viral RNA using the one-step RT-LAMP method, the RT-LAMP mix contained the following: 20 μ L RM, 2 μ L BSA (20 mg/mL), 2 μ L EM, 2 μ L FD, 2 μ L of primer mixture (20 μ M BIP/FIP, 10 μ M LooP_B/Loop_F and 2.5 μ M B3/F3), various amount of template solution, and enough nuclease-free water bring the volume to 40 μ L. The solution was loaded onto a SlipChip and heated at 63 °C for 60 minutes.

To amplify HIV viral RNA using the two-step RT-LAMP method, the first solution contained the following: 10 μ L RM, 1 μ L BSA, 0.5 μ L EXPRESS SYBR® GreenER™ RT module (part of EXPRESS One-Step SYBR® GreenER™ Universal), 0.5 μ L BIP primer (10 μ M), various amount of, and enough nuclease-free water to bring the volume to 20 μ L. The second solution contained: 10 μ L RM, 1 μ L BSA, 2 μ L DNA polymerase solution (from LoopAmp® DNA amplification kit), 1 μ L or 2 μ L FD, 2 μ L other primer mixture (20 μ M FIP, 17.5 μ M FIP, 10 μ M

LooP_B/Loop_F and 2.5 μ M F3), 1 μ L Hybridase™ Thermostable RNase H, and enough nuclease-free water to bring the volume to 20 μ L. The first solution was loaded onto a SlipChip device and incubated at 37 °C or 50 °C, then the second solution was loaded onto the same device and mixed with the first solution, and the entire device was incubated at 63 °C for 60 minutes.

To amplify λ -DNA, the LAMP mix contained the following: 20 μ L RM, 2 μ L BSA (20 mg/mL), 2 μ L DNA polymerase, 2 μ L FD, 2 μ L of primer mixture (20 μ M BIP/FIP, 10 μ M LooP_B/Loop_F and 2.5 μ M B3/F3), various amount of template solution, and enough nuclease-free water to bring the volume to 40 μ L. The same loading protocol as above was performed and the device was incubated at 63 °C for 70 minutes.

To amplify ssDNA, the LAMP mix contained the following: 20 μ L RM, 2 μ L BSA, 2 μ L DNA polymerase, 2 μ L FD, 2 μ L of primer mixture (20 μ M BIP/FIP, 10 μ M LooP_B/Loop_F and 2.5 μ M B3/F3), various amount of template solution, and enough nuclease-free water to bring the volume to 40 μ L. The same loading protocol as above was performed and the device was incubated at 63 °C for 60 minutes.

dRT-PCR amplification on SlipChip

To amplify HIV viral RNA, the RT-PCR mix contained the following: 20 μ L 2X Evagreen SuperMix, 2 μ L BSA, 1 μ L EXPRESS SYBR® GreenER™ RT module, 1 μ L each primer (10 μ M), 2 μ L template, and enough nuclease-free water to bring the volume to 40 μ L. The amplification was performed at the same conditions as reported before⁹ except for a shortened reverse transcription step of 10 minutes.

Image acquisition and analysis

The bright-field image and the fluorescence images in real-time dRT-LAMP experiments were acquired using a Leica MZ 12.5 Stereomicroscope. All other fluorescence images were acquired

using a Leica DMI 6000 B epi-fluorescence microscope with a 5X / 0.15 NA objective and L5 filter at room temperature unless stated otherwise. All the images were analyzed using MetaMorph software (Molecular Devices, Sunnyvale, CA).

APPENDIX D

Authorship contribution statement and experimental section for Chapter 5

In the following publication

Selck, D. A.; Karymov, M. A.; Sun, B.; Ismagilov, R. F., Increased Robustness of Single-Molecule Counting with Microfluidics, Digital Isothermal Amplification, and a Mobile Phone versus Real-Time Kinetic Measurements. *Anal. Chem.* **2013**, *85* (22), 11129-11136.

D.A.S, M.A.K. and B.S. contributed equally to this work.

D.A.S, M.A.K. and B.S. and R.F.I. designed the experiments and wrote the paper.

D.A.S performed all cell phone image acquisition with M.K., developed the software for data analysis, and assisted B.S. for in digital experiments.

B.S. performed all digital experiments and data acquisition with microscope analysis with Metamorph software.

M.K. was the lead researcher on Figure 3 and assisted cell phone image acquisition.

Experimental section (as described in the publication above)

Chemicals and materials

All solvents and salts purchased from commercial sources were used as received unless otherwise stated. The LoopAmp® DNA amplification kit, the LoopAmp® RNA amplification kit, and the Calcein fluorescence indicator kit were purchased from SA Scientific (San Antonio, TX, USA). SsoFast EvaGreen Supermix (2X) was purchased from Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin (BSA) was purchased from Roche Diagnostics (Indianapolis, IN). Hybridase™ Thermostable RNase H was purchased from Epicentre Biotechnologies (Madison, WI). All primers were ordered from Integrated DNA Technologies (Coralville, IA). Mineral oil (DNase-, RNase-, and Protease-free), tetradecane, and DEPC-treated nuclease-free water were purchased from Fisher Scientific (Hanover Park, IL). Dichlorodimethylsilane was purchased from Sigma-Aldrich (St. Louis, MO). AcroMetrix® HIV-1 Panel Copies/ml, EXPRESS One-Step SYBR® GreenER™ Universal, iPrep™ purification instrument, and iPrep™ PureLink virus kit were purchased from Life Technologies (Grand Island, NY). PCR Mastercycler was purchased from Eppendorf (Hamburg, Germany). Eco real-time PCR system was purchased from Illumina, Inc. (San Diego, CA). Photomasks were designed in AutoCAD 2013 and ordered from CAD/Art Services, Inc. (Bandon, OR). Soda-lime glass plates coated with layers of chromium and photoresist were ordered from the Telic Company (Valencia, CA). Genomic DNA (*Staphylococcus aureus*, ATCC number 6538D-5) was purchased from American Type Culture Collection (Manassas, VA).

Fabrication, assembling and loading of SlipChip

The fabrication, assembling and loading protocol was the same as described in Appendix C.

Primers used for RT-LAMP amplification

F3	ATTATCAGAAGGAGCCACC
B3	CATCCTATTTGTTTCCTGAAGG
FIP	CAGCTTCCTCATTGATGGTCTCTTTTAACACCCATGCTAAACACAGT
BIP	TATTGCACCAGGCCAGATGATTTTGTACTAGTAGTTCCTGCTATG
LOOPF	TTTAACATTTGCATGGCTGCTTGAT
LOOPB	GAGAACCAAGGGGAAGTGA

dRT-LAMP amplification of HIV-1 RNA on SlipChip

The first solution, which was used for amplifying HIV-1 RNA using the two-step dRT-LAMP method, contained the following: 10 μ L RM, 1 μ L BSA, 0.5 μ L EXPRESS SYBR® GreenER™ RT module (part of EXPRESS One-Step SYBR® GreenER™ Universal), 0.5 μ L BIP primer (10 μ M), various amounts of template, and enough nuclease-free water to bring the volume to 20 μ L. The second solution contained 10 μ L RM, 1 μ L BSA, 2 μ L EM (from LoopAmp® RNA amplification kit), 1 μ L or 2 μ L FD, 2 μ L other primer mixture (20 μ M FIP, 17.5 μ M FIP, 10 μ M Loop_B/Loop_F, and 2.5 μ M F3), 1 μ L Hybridase™ Thermostable RNase H, and enough nuclease-free water to bring the volume to 20 μ L. The first solution was loaded onto a SlipChip device and incubated at 50 °C for 10 min, and then the second solution was loaded onto the same device and mixed with the first solution. The entire filled device was incubated at various temperatures (57 °C, 60 °C, or 63 °C) for 60 minutes.

Real-time RT-LAMP amplification of HIV-1 RNA

For two-step RT-LAMP amplification, a first solution containing the reagents described above was first incubated at 50 °C for 10 min and then mixed with a second solution, as described above. The 40 μ L mixture was split into 4 aliquots and loaded onto an Eco real-time PCR machine. The amplification was performed at various temperatures (57 °C, 60 °C, or 63 °C) for 60 min. For one-step RT-LAMP amplification, the RT-LAMP mix contained the following: 20 μ L RM, 2 μ L

BSA (20 mg/mL), 2 μ L EM, 2 μ L FD, 2 μ L of primer mixture (20 μ M BIP/FIP, 10 μ M LooP_B/Loop_F, and 2.5 μ M B3/F3), various amount of template solution, and enough nuclease-free water bring the volume to 40 μ L. The 40 μ L mixture was split into 4 aliquots and loaded onto the Eco real-time PCR machine. The amplification was performed at various temperatures (57 $^{\circ}$ C, 60 $^{\circ}$ C, or 63 $^{\circ}$ C) for 60 min. Data analysis was performed using Eco software. To determine the reaction time (time required for the fluorescent signal to cross the threshold), fluorescence intensity between 5 min and 15 min was used as the baseline and the threshold value was set to be 2.

Microscope image acquisition and analysis

Fluorescence images of each device were acquired using a Leica DMI 6000 B epi-fluorescence microscope with a 5X / 0.15 NA objective and L5 filter at room temperature. All the images were analyzed using MetaMorph software (Molecular Devices, Sunnyvale, CA). Images taken in each experiment were stitched together and a dark noise background value of 110 units was subtracted before the image was thresholded. The number of positive wells was automatically counted using the integrated morphology analysis tool based on intensity and pixel area. Typical arbitrary fluorescence values for the negative wells were 80 ± 10 . Arbitrary fluorescence values for the positive wells were largely centered around 350 ± 100 .

Cell phone camera setup and settings

A Nokia 808 Pureview cell phone was used to image and count microwells containing the amplification product. This cell phone features a CMOS sensor with a Xenon flash. The Nokia 808 uses a 1/1.4-inch 41-megapixel sensor with a pixel size of 1.4 μ m. The camera has a Carl Zeiss F2.4 8.02 mm lens and contains a set of “PureView” modes which are able to combine multiple pixels by using pixel oversampling, thus increasing an individual pixel’s area (which, consequently, lowers the image’s resolution). This feature increases the sensitivity of each

individual pixel in the final image. Since the shortest focal length of the camera's lens is 15 cm, we used a non-branded, commercially available 0.67 x magnetically mounted wide lens designed for iPhone. Using this objective, we were able to obtain images at a distance of 6.5 cm from the device, which effectively increased the total number of photons collected from the device, thus improving camera sensitivity. A set of filters was used for both excitation and emission purposes. To improve filtering, one additive dichroic filter (model number FD1B, Thorlabs, Newton, NJ) was cut in half and the two halves were stacked and attached to the camera. For fluorescence detection, two 5CGA-530 long-pass filters (Newport, Franklin, MA) were inserted into magnetically mounted lens. The imaging process with a cell phone was performed with the device tilted at 10 degrees relative to the cell phone plane to prevent direct reflection of the flash to the lens. This was achieved by placing the device on a tilted glass slide (3" x 2") that had been painted black on the bottom side to reduce background noise. Tetradecane was placed in between the device and the slide to reduce glare. All images were taken using the stock camera application. The white balance was set to automatic, the ISO was set at 800, the exposure value was set at +2, the focus mode was set to "close-up," and the resolution was adjusted to 8 MP.

Real-time dRT-LAMP imaging and analysis

Time traces of dRT-LAMP reactions were obtained by imaging a progressing dRT-LAMP reaction every minute using a Leica MZFLIII stereomicroscope. This was performed by placing an Eppendorf mastercycler with an in-situ adapter plate within the imaging field of the stereoscope. The stereoscope was set to a magnification of 1X and placed in such a way that it could move relative to the position of the thermocycler. At each required time-point, a fluorescent image of one-half of the device was taken, and then the stereoscope was moved to image the second half of the device. The camera attached to the stereoscope was a Diagnostic Instruments color mosaic model 11.2 camera and images were acquired using Spot imaging software. The imaging was done with a gain of 4 and an exposure time of 2 seconds. The acquired images were

then analyzed using the ImageJ software package. The images were manually edited to remove wells that appeared in both images, after which, the green color plane was extracted and a threshold value of 30 was applied. The binary image was then filtered to keep all spots within an area between 65 and 350 leaving the number of positive wells. After performing this analysis with images comprising both halves of the device, the numbers for each half were added together, yielding the total number of positive wells at that specific time point.

Statistical analysis of data sets obtained at different temperatures

The t-test is used to evaluate whether the means of two different data sets are statistically different. The p value obtained in this process is the probability of obtaining a given result assuming that the null hypothesis is true. A 95% confidence level, which corresponds to $p = 0.05$, or a 5% significance level, is commonly acceptable. It is typically assumed that the concentrations of two samples are different when $p < 0.05$. Here, we used a p value to evaluate the performance of two-step dRT-LAMP in various imaging conditions—with a microscope, with a cell phone and a shoe box, and with a cell phone in dim lighting. When we pooled all data for one concentration from different temperatures and compared them to data acquired at another concentration, the highest p value among the three imaging conditions was 6.7×10^{-7} . Thus, the two concentrations were clearly distinguishable and the null hypothesis, which stated that both concentrations were equivalent, was rejected. We also compared the two closest subsets (2×10^5 copies/mL at 57 °C and 1×10^5 copies/mL at 63 °C) and calculated their p-value under each set of imaging conditions. The p-values were still below 0.05 for all three conditions. Additionally, we used normal distributions as visual guides for data interpretation. We can use normal distributions instead of theoretical t-distributions because we determined standard deviations from the data. There was no visible overlap between the data sets corresponding to the two concentrations.

Cloud-based automatic analysis

The Symbian software on which the Nokia 808 cell phone is based can access Skydrive, a cloud-based storage service produced by Microsoft. This service can automatically upload images to the cloud for storage directly after imaging, without any user intervention. Almost instantly, those uploaded files are synced with all other computers running Skydrive that are logged in to the same account, and the images can be analyzed on multiple computers. Here, we used a secondary computer with a custom Labview program, including a built-in “file watcher” function whereby all files added to a specific directory that fell within a specific filtered category (i.e., *.jpg) were automatically analyzed. The analysis of images included a multi-step algorithm. First, the RGB color image was split into three monochrome 8-bit images for each individual color. The red-channel image was used to determine whether or not the entire chip had been imaged by searching for markers on the device (four red circles of tape, in this case). If all circles had been found, the image was then rotated such that the device was parallel to the top of the image box, removing any rotational bias. A background-corrected image was then generated by subtracting the red-channel monochrome image from the green-channel monochrome image, which contained the fluorescence information. The image was then subjected to a filtering process to increase the intensity of the positive wells. The filtering process included the following steps, in the following order: i) a 3 x 3 “local average” filter, ii) a 2 x 2 “median” filter, iii) an 11 x 11 “highlight details” filter, and iv) a 5 x 5 “median” filter. The filtered image was then thresholded using an entropy algorithm. After thresholding, a portion of the image (defined by the position of the markers) was analyzed and all individual spots were subjected to a size-filtering algorithm. This yielded the eventual total number of counts, which was then statistically transformed into a concentration before being emailed to the user or proper authority.

APPENDIX E

Authorship contribution statement and experimental section for Chapter 6

In the following publication

Bing Sun, Jesus Rodriguez-Manzano, David A. Selck, Eugenia Khorosheva, Mikhail A. Karymov, and Rustem F. Ismagilov, "Measuring Fate and Rate of Single-Molecule Competition of Amplification and Restriction Digestion, and Its Use for Rapid Genotyping Tested with Hepatitis C Viral RNA" *Angew. Chem. Int. Ed.* **2014**, accepted.

B.S, J.R.M and R.F.I developed the idea, designed the experiments and prepared the manuscript.

B.S and J.R.M contributed equally.

B.S and J.R.M led and performed the experiments and the data analysis (Figure 17 to Figure 21 in this dissertation).

D.A.S built the real-time digital instrument and helped image enhancement.

E.K and M.A.K performed the HCV RT-LAMP primer design and optimization based on previous publication, which was further optimized by J.R.M.

Experimental section (as described in the publication above)

Chemicals and materials

All solvents and salts purchased from commercial sources were used as received unless otherwise stated. The Loopamp® RNA amplification kit and the Loopamp® Fluorescent Detection Reagent kit were purchased from SA Scientific (San Antonio, TX, USA). The LoopAmp® RNA amplification kit contains 2X Reaction Mix (RM) (40 mM Tris-HCl pH 8.8, 20 mM KCl, 16 mM MgSO₄, 20 mM (NH₄)₂SO₄, 0.2% Tween20, 1.6 M Betaine and dNTPs 2.8 mM each), Enzyme Mix (EM) (mixture of Bst DNA polymerase and AMV reverse transcriptase), and distilled water (DW). Loopamp® Fluorescent Detection Reagent kit contains Fluorescent Detection Reagent (FD) (including calcein). SsoFast EvaGreen Supermix (2X) was purchased from Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin (BSA) was purchased from Roche Diagnostics (Indianapolis, IN). All restriction enzymes were purchased from New England Biolabs (Ipswich, MA). All primers were ordered from Integrated DNA Technologies (Coralville, IA). Mineral oil (DNase, RNase, and Protease free), tetradecane, and DEPC-treated nuclease-free water were purchased from Fisher Scientific (Hanover Park, IL). Dichlorodimethylsilane was purchased from Sigma-Aldrich (St. Louis, MO). AcroMetrix® HCV-s panel and AcroMetrix® HCV High Control and EXPRESS One-Step SYBR GreenER Universal were purchased from Life Technologies (Grand Island, NY). Genotype 3 and genotype 4 HCV viral isolates were purchased from SeraCare Life Sciences (Gaithersburg, MD). Nucleic acid extraction kit QIAamp Viral RNA Mini kit was purchased from QIAGEN (Inc, Valencia, CA, USA) PCR Mastercycler and in situ adapter were purchased from Eppendorf (Hamburg, Germany). Eco real-time PCR system was purchased from Illumina, Inc. (San Diego, CA). Photomasks were designed in AutoCAD 2013 and ordered from CAD/Art Services, Inc. (Bandon, OR). Soda-lime glass plates coated with layers of chromium and photoresist were ordered from the Telic Company (Valencia,

CA). Sanger sequencing service was provided by Laragen, Inc. HCV sequences were extracted from Los Alamos database and aligned with Geneious software.

HCV isolates in plasma

We assayed 4 different HCV genotypes. Genotypes 1 and 2 were purchased from Acrometrix Corporation (Benicia, CA, USA) and genotypes 3 and 4 from SeraCare Life Sciences (Milford, MA, USA). Genotype and viral load information was provided by these companies: viral load of 2.5×10^7 IU/mL for GT1, $1.1 \times 10^6 \sim 3.4 \times 10^6$ IU/mL for GT2, 5.7×10^6 IU/mL for GT3, and 4.97×10^6 IU/mL for GT4. The genotype information was also provided by the companies and we confirmed the genotype by sequencing and phylogenetic analysis. The presence of targeted single-nucleotide polymorphisms or SNPs (restriction enzyme cutting sites) was confirmed by manual inspection of the sequencing chromatograms.

RNA extraction from HCV isolates

RNA was extracted using the QIAamp Viral RNA Mini Kit (QIAGEN Inc, Valencia, CA, USA) according to the manufacturer's instructions, using 200 μ L of plasma and eluting the resulting nucleic acid extraction in 60 μ L of elution buffer. Nucleic acid extractions were analyzed immediately or stored at -80 °C until further analysis.

Preparing DNA for sequencing using RT-PCR

To amplify HCV viral RNA, the RT-PCR mix contained the following: 20 μ L of 2X SsoFast Evagreen SuperMix, 1 μ L of EXPRESS SYBR GreenER RT module, 1 μ L of each primer (10 μ M), 2 μ L of template, and enough nuclease-free water to bring the volume to 40 μ L. The reverse transcription was carried out at 50 °C for 15 min, followed by 2 min of reaction termination at 95 °C. The amplification step was performed by 40 cycles of the following conditions: 95 °C for 15 seconds, 55 °C for 1 min and 72 °C for 1 min.

Phylogenetic analysis

The dendrogram was constructed by alignment of the 222 nucleotide sequences within the UTR region of HCV based on the UPGMA method under the Tamura-Nei model (bootstrap = 1,000 replicates). Reference sequences from HCV strains (genotypes 1 to 4) were obtained from the Los Alamos HCV database.

Primers used for RT-LAMP

F3	CCTCCCGGGAGAGCCATAG;
B3	GCACTCGCAAGCACCCITATC
FIP	TCCAAGAAAGGACCCIGTCTTTTTCTGCGGAACCGGTGAGTAC
BIP	TTGGGCGTGCCCCGCIAGATTTTTCAGTACCACAAGGCCITTCGCIACC
LOOPF	TTICCGGIAATTCCGGT
LOOPB	CTGCTAGCCGAGTAGIGTTG

Real-time bulk RT-LAMP/RE competition assay

To amplify HCV viral RNA using RT-LAMP on a real-time PCR machine, the RT-LAMP mix contained the following: 20 μ L of 2X reaction mix (RM), 2 μ L of enzyme mix (EM), 1 μ L of fluorescent detection reagent (FD), 4 μ L of primer mixture (20 μ M BIP/FIP, 10 μ M Loop_B/Loop_F, and 2.5 μ M B3/F3), various amounts of RNA template solution, and enough nuclease-free water to bring the volume to 40 μ L. The solution was split into 10 μ L each and loaded into 3 wells on the Eco real-time PCR plate and heated at 63 °C for 50 min. RT-LAMP reagents and FD were used as purchased from SA Scientific and used as it was.

To amplify HCV viral RNA using RT-LAMP in the presence of RE on real-time PCR machine, the RT-LAMP mix contained the following: 20 μ L of RM, 2 μ L of EM, 1 μ L of FD, 4 μ L of primer mixture (20 μ M BIP/FIP, 10 μ M Loop_B/Loop_F, and 2.5 μ M B3/F3), various amounts

of RNA template solution, 4 μL 20-fold diluted RE (to make a 200-fold dilution in the final solution) and enough nuclease-free water to bring the volume to 40 μL . RE was diluted in nuclease-free water before immediately mixed with RT-LAMP reagents, and fresh dilution was made each time. The solution was split into 10 μL each and loaded into 3 wells on the Eco real-time PCR plate and heated at 63 $^{\circ}\text{C}$ for 50 min.

Real-time bulk RT-LAMP/RE data analysis

Bulk RT-LAMP/RE assays were carried out in an Eco Real-Time PCR System (Illumina, SD, USA) and data analysis was performed using Eco Real-Time PCR System Software (version 4.0). To determine the time-to-positive (time required for the fluorescent signal to cross the threshold), fluorescence intensity between 5 min and 15 min was used as the baseline and the threshold value was set to be half height of the maximum intensity.

Fabrication of SlipChip

The single-volume 1280-well SlipChip was designed and optimized based on previous work. The procedure of fabricating SlipChip from soda-lime glass was based on the procedure described in previous work. All features were etched to a depth of 55 μm to make the volume of loading well equal to 3 nL. The cleaning, assembling and loading protocol was same as described in other chapters.

Real-time digital competition assay on SlipChip

To amplify HCV viral RNA using RT-LAMP method on real-time PCR machine, the RT-LAMP mix contained the following: 20 μL of RM, 2 μL of EM, 1 μL of FD, 4 μL of primer mixture (20 μM BIP/FIP, 10 μM Loop_B/Loop_F, and 2.5 μM B3/F3), 2 μL of BSA (20 mg/mL), various amounts of RNA template solution, 4 μL diluted RE if not for positive control, and enough nuclease-free water to bring the volume to 40 μL . The solution was loaded onto SlipChip and

heated at 63 °C for 50 min on a custom-built real-time instrument. RT-LAMP reagents and FD were used as purchased from SA Scientific. BSA was used as purchased from Roche Diagnostics.

Custom-built real-time instrument imaging

Experiments were performed on a Bio-Rad PTC-200 thermocycler with a custom machined block. The block contains a flat 3" x 3" portion onto which the devices are placed ensuring optimal thermal contact. The excitation light source used was a Philips Luxeon S (LXS8-PW30) 1315 lumen LED module with a Semrock filter (FF02-475). Image Acquisition was performed with a VX-29MG camera and a Zeiss Macro Planar T F2-100mm lens. A Semrock filter (FF01-540) was used as an emission filter.

Data analysis using Labview software

Images acquired were analyzed using self-developed Labview software. The data were analyzed by first creating a binary mask that defined the location of each reaction volume within the image. The masked spots were then overlaid on the stack of images collected over the course of the experiment and the average intensity of each individual masked spot was tracked over the course of the stack. Background subtraction of the real-time trace was performed by creating a least mean square fit of each individual trace. Threshold was then manually set at the half height of the averaged maximum intensity, and the time-to-positive of each reaction was then determined as the point at which the real-time curve crossed the defined threshold.

Cell phone imaging protocol

Cell phone imaging setup was the same as previously described. The white balance was set to automatic, the ISO was set at 800, the exposure value was set at +2, the focus mode was set to "close-up", and the resolution was adjusted to 8 MP.

DNA gel electrophoresis analysis of RE digestion of amplified RT-LAMP product

To test the specificity and activity of RE at the condition for RT-LAMP, we first prepared RT-LAMP product from HCV RNA of genotype 1, 2, 3, and 4, respectively. The amplification procedure was the same as described in “Real-time bulk RT-LAMP/RE competition assay” except that an additional 5 min at 85 °C was used to inactivate the polymerase. 2 µL RT-LAMP product was mixed with 4 µL fresh RM, 3 µL nuclease-free water and 1 µL RE (or water for non-RE control) and incubated at 63 °C for 30 min. The digestion product was analyzed on 1.2% agarose DNA gel stained with ethidium bromide at 75 Volt for 40 min.

APPENDIX F

Poisson statistics in the context of digital PCR.

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Poisson analysis of single-volume PCR:

There are two key assumptions that need to be maintained in order for digital PCR methods and analysis to accurately quantify nucleic acid concentrations: 1) Having at least one target molecule in a well is necessary and sufficient for a positive signal and 2) Target molecules do not interact with one another or device surfaces, to avoid biasing their distribution. At the simplest level of analysis, when molecules are at low enough densities that there is either 0 or 1 molecule within a well, concentrations can be estimated simply by counting positives. However, if the two key assumptions above hold true, then Poisson and binomial statistics can be used to obtain quantitative results from experiments resulting in one positive well to experiments resulting in one negative well. The Poisson distribution (Eq. 1), in the context of digital PCR, gives the probability, p , that there are k target molecules in a given well based on an average concentration per well, $v \cdot \lambda$, where v is the well volume (mL) and λ is the bulk concentration (molecules/mL). In digital PCR, the same readout occurs for all $k > 0$, so if $k=0$ then Eq. 1 simplifies to give the probability, p , that a given well will not contain target molecules (the well is “negative”).

$$p = \left((v \cdot \lambda)^k \cdot e^{-(v \cdot \lambda)} \right) / k!, \text{ and for } k=0 \text{ (empty well), } p = e^{-(v \cdot \lambda)} \quad (1)$$

In single-volume systems, the number of negative wells, b , out of total wells, n , can serve as an estimate for p , so expected results can be estimated from known concentrations, or observed results can be used to calculate expected concentrations (Eq. 2).

$$b = n \cdot e^{-(v \cdot \lambda)} \text{ or } \lambda = -\ln(b/n)/v \quad (2)$$

The binomial equation is used to determine the probability, P , that a specific experimental result (with a specific number of negatives, b , and positives, $n-b$, out of the total number of wells, n , at each volume) will be observed, based on λ (Eq. 3), where

$$\binom{n}{b} = \frac{n!}{b!(n-b)!}.$$

$$P = \binom{n}{b} \cdot p^b \cdot (1-p)^{n-b} \text{ or } P = \binom{n}{b} \cdot (e^{-v \cdot \lambda})^b \cdot (1 - e^{-v \cdot \lambda})^{n-b} \quad (3)$$