Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

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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**

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Supporting Information

Experimental section

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 $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 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, 1.1×10^6~3.4×10^6 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 μL of plasma and eluting the resulting nucleic acid extraction in 60 μL 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 μL of 2X SsoFast Evagreen SuperMix, 1 μL of EXPRESS SYBR GreenER RT module, 1 μL of each primer (10 μM), 2 μL of template, and enough nuclease-free water to bring the volume to 40 μL. 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 dendogram was constructed by alignment of the 222 nucleotide sequences within the 5'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.

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 μL of 2X reaction mix (RM), 2 μL of enzyme mix (EM), 1 μL of fluorescent detection reagent (FD), 4 μL of primer mixture (20 μM BIP/FIP, 10 μM LB/LF, and 2.5 μM B3/F3), various amounts of RNA template solution (2.86 μL GT1 RNA for Figure 3, 2 μL 10-fold diluted GT1 RNA and 2 μL RNA for all the other genotypes for figure 4 and 5), and enough nuclease-free water to bring the volume to 40 μL. The solution was split into 10 μL 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 μL of RM, 2 μL of EM, 1 μL of FD, 4 μL of primer mixture (20 μM BIP/FIP, 10 μM LB/LF, and 2.5 μM B3/F3), various amounts of RNA template solution (2.86 μL GT1 RNA for Figure 3, 2 μL 10-fold diluted GT1 RNA and 2 μL RNA for all the other genotypes for figure 4 and 5), 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 °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.[1] The procedure of fabricating SlipChip from soda-lime glass was based on the procedure described in previous work.[2] 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 previously described.[2]

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 LB/LF, and 2.5 μM B3/F3), 2 μL of BSA (20 mg/mL), various amounts of RNA template solution (2.86 μL GT1 RNA for Figure 3, 2 μL 10-fold diluted GT1 RNA and 2 μL RNA for all the other genotypes for figure 4 and 5), 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.[3] 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.

Determining optimal RE concentration
To determine the restriction enzyme concentration which did not trigger ab initio synthesis within 50 min, three dilutions (100-fold, 200-fold and 300-fold dilution in the final mixture) of each RE were added to RT-LAMP mix containing the same components as in the genotyping assay with the exception of HCV RNA template that was replaced with nuclease-free water. 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 °C for 96 min.

Commercially available HCV genotyping assays
High-complexity molecular tests such as commercially available HCV genotyping assays are not well suited for limited-resource settings: for example, hybridization assays (Roche LINEAR ARRAY Hepatitis C Virus Genotyping Test, Siemens VERSANT HCV Genotype 2.0 assay (LiPA)) and hybridization followed by electrochemical readout (GenMark eSensor) assays start with a PCR step, take from several hours up to one day, and require strict control of conditions; sequencing analysis is also slow and requires complex protocols and instrumentation (TRUGENE HCV Genotype Test). Automated real-time RT-PCR with Taqman probes (Abbott RealTime HCV Genotype II) is faster (~ 5 hrs) but is still too complex and as a kinetic measurement not sufficiently robust for limited-resource settings.

Figures and tables

Table S1 Sequence of primers used in RT-LAMP.

primer	sequence (5'-3')
F3	CCTCCCGGGAGAGCCATAG
FIP	TCCAAGAAAGGACCCIGTCTTTTTCTGCGGAACCGGTGAGTAC
LF	TTICCGGIAATTCCGGT
B3	GCACTCGCAAGCACCITATC
BIP	TTGGGCGTGCCCCCGCIAGATTTTTCAGTACCACAAGGCCITTCGCIACC
LB	CTGCTAGCCGAGTAGIGTTG

Table S2 Sequencing results for HCV RNA purified from purchased isolates.

Genotype 1	TCGTGCAGCCTCCAGGACCCCCCCTCTCGGGAGAGCCATAGTGGTCTGCGGAAC CGGTGAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCTTGGATCAACCCG CTCAATGCCTGGAGATTTGGGCGTGCCCCCGCGAGACTGCTAGCCGAGTAGTGT TGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCTCG GGAGGT
Genotype 2	TCGTACAGCCTCCAGGCCCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAAC CGGTGAGTACACCGGAATTGCCGGGAAGACTGGGTCCTTTCTTGGATAAACCCA CTCTATGCCCGGCCATTTGGGCGTGCCCCCGCAAGACTGCTAGCCGAGTAGCGT TGGGTTGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCG GGAGGT
Genotype 3	TCGTGCAGCCTCCAGGATCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAAC CGGTGAGTACACCGGAATCGCTGGGGTGACCGGGTCCTTTCTTGGAGCAACCCG CTCAATACCCAGAAATTTGGGCGTGCCCCCGCGAGATCACTAGCCGAGTAGTGT TGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCG GGAGGT
Genotype 4	TTGTACAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAAC CGGTGAGTACACCGGAATCGCCGGGATGACCGGGTCCTTTCTTGGATAAACCCG CTCAATGCCCGGAAATTTGGGCGTGCCCCCGCAAGACTGCTAGCCGAGTAGTGT TGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCG GGAGGT

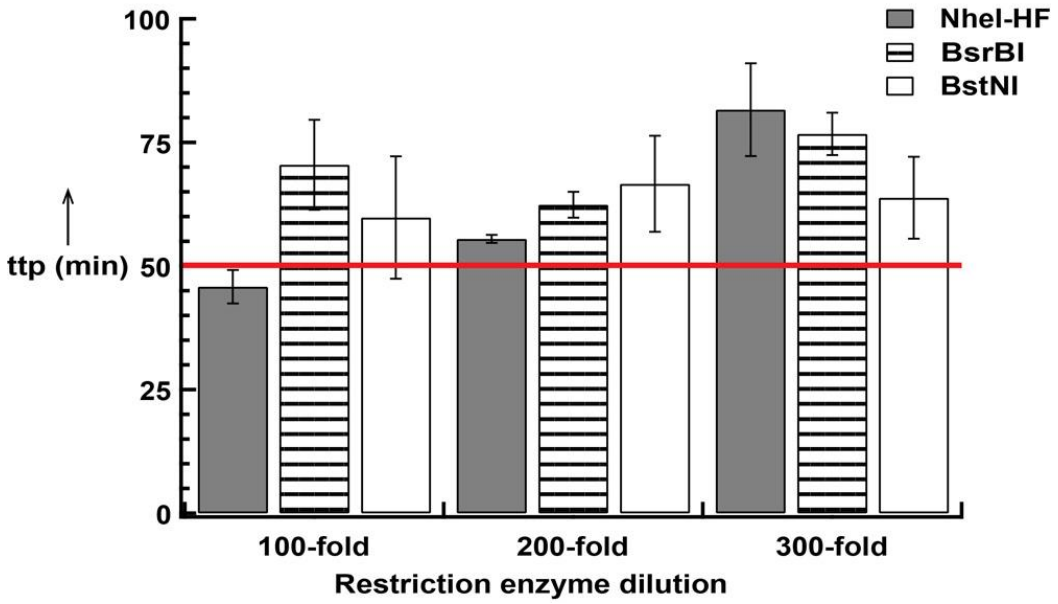

Figure S1 Results of real-time RT-LAMP negative controls with different dilutions of RE. Columns represent time to positive (ttp) caused by ab initio DNA synthesis and error bars stand for standard deviation. RT-LAMP reactions were performed in the absence of HCV RNA and in the presence of three different dilutions (100-fold, 200-fold and 300-fold) of NheI (grey), BsrBI (striped), and BstNI (white). Horizontal red line shows the ttp threshold set for analysis (50 min). In the experiments reported in this paper, 200-fold RE dilution was used because 100-fold dilution was not enough to remove the influence of ab initio DNA synthesis within 50 min.

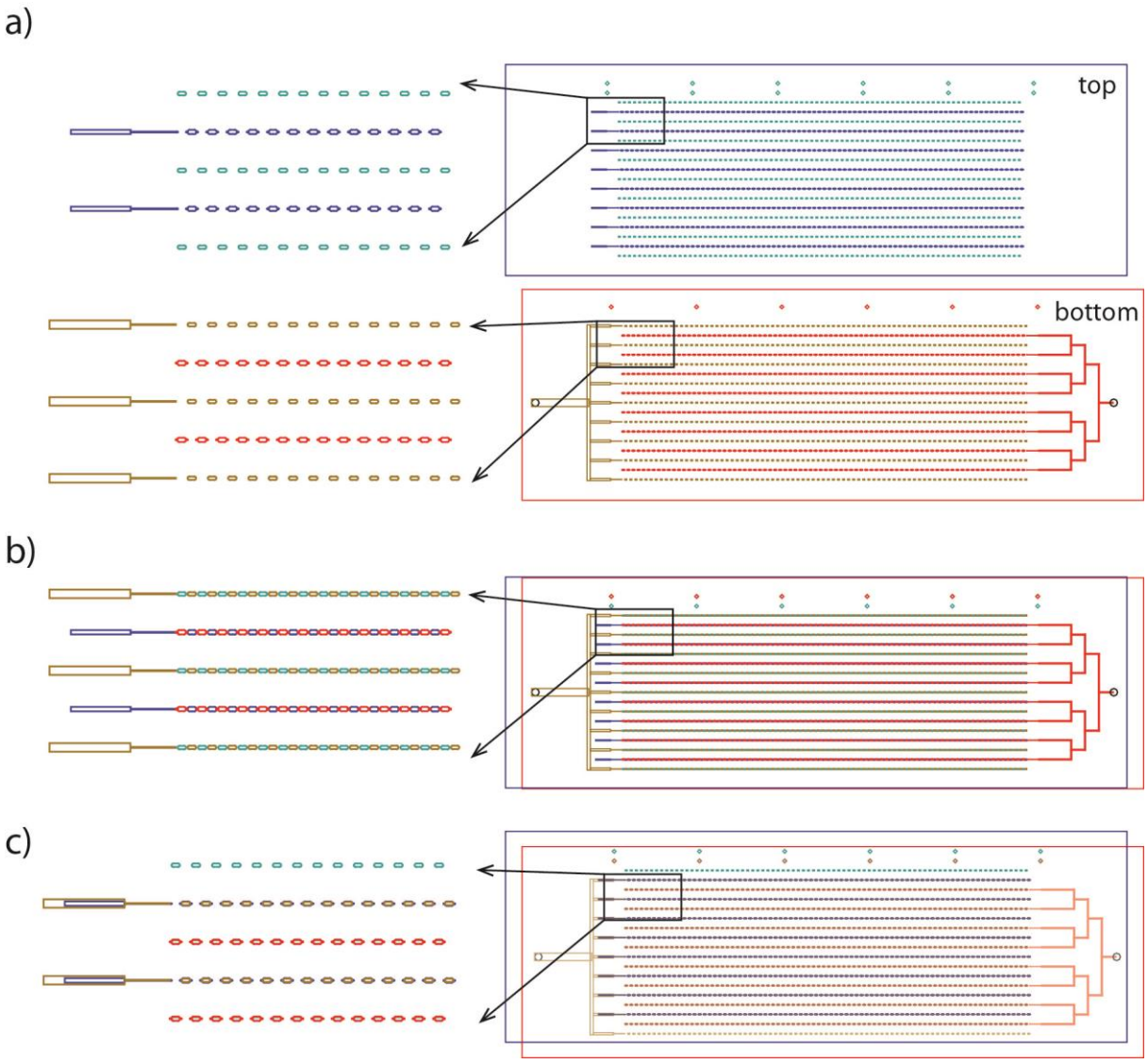

Figure S2 Layout of the 1280-well SlipChip device used in the digital competition assay. Schematic drawings show a) the layout of top and bottom piece of the entire device on the right and a zoomed-in region (black box) on the left, b) the relative position of two pieces when they are aligned to allow the loading of solution through the channel, and c) the relative position of two pieces when they are slipped to separate droplets from one another and form compartments.

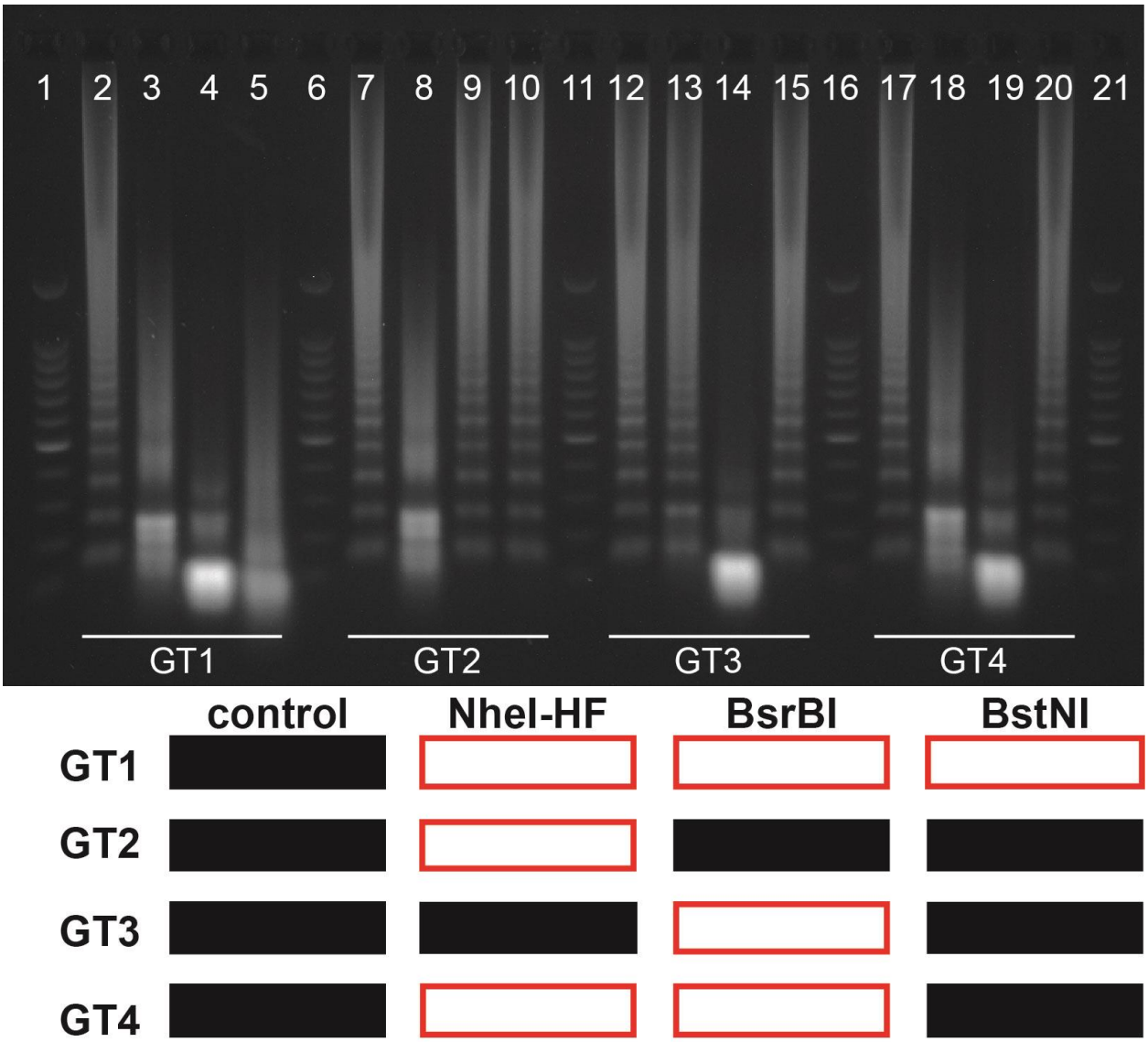

Figure S3 DNA gel electrophoresis for digestion of RT-LAMP product at RT-LAMP conditions and predicted HCV genotyping pattern. Top: Image of DNA gel electrophoresis results for digestion of RT-LAMP product at RT-LAMP conditions. Lanes 1, 6, 11, 16 and 21 are 100 bp DNA ladders. Lanes 2-5 are positive control, NheI-HF digestion product, BsrBI digestion product, and BstNI digestion product for genotype 1, respectively. Lanes 7-10 are positive control and 3 RE digestion products for genotype 2. Lanes 12-15 are for genotype 3 and Lanes 17-20 for genotype 4. The specificity of RE to different genotypes are the same as predicted in Figure 4a: for genotype 1 all 3 REs digested the product; for genotype 2 only NheI-HF digested the product; for genotype 3 only BsrBI digested the product, and for genotype 4 both NheI-HF and BsrBI digested the product. Bottom: Figure 4a from the main text (included as a reference), which shows the predicted HCV genotyping pattern based on REs used.

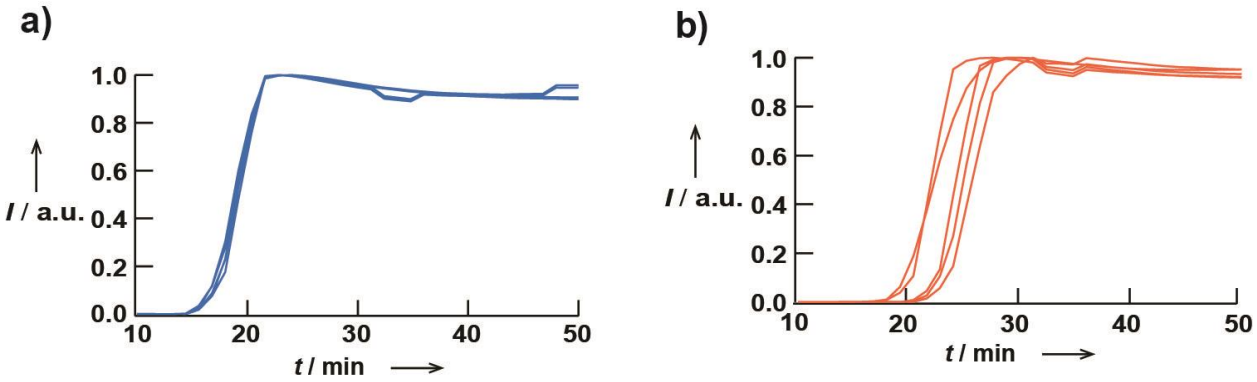

Figure S4 Results of real-time RT-LAMP performed in bulk for GT1 and BsrBI. a) Real-time RT-LAMP curves for GT1 in the absence of RE (positive control). b) Real-time RT-LAMP curves for GT1 in the presence of BsrBI.

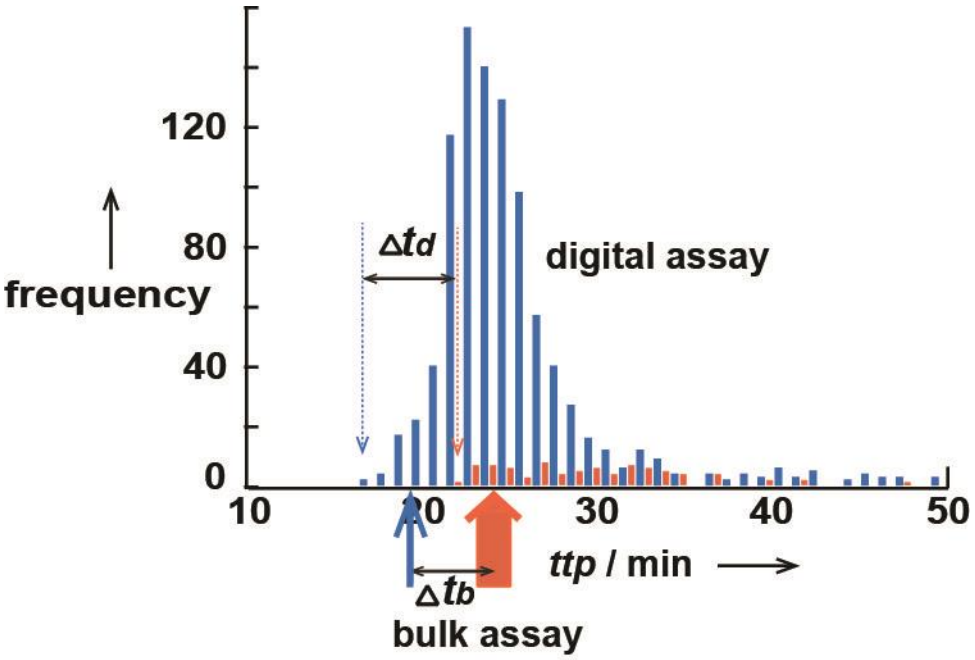

Figure S5 Histogram of real-time, single-molecule digital RT-LAMP/RE experiments for HCV GT1 RNA. Graph shows the change of rate for positive wells in the absence of RE (blue) and in the presence of BsrBI (red). The two bars below the x-axis show time-to-positive for real-time bulk experiments, the widths of which stand for standard deviation for the bulk assay (n=5).

Table S3 Statistical analysis of digital counts obtained in positive controls for RT-LAMP of different HCV genotypes. Distribution of numbers of amplifiable RNA molecules in wells calculated based on observed digital counts and Poisson statistics.

HCV RNA	GT1	GT2	GT3	GT4
Average counts	190	96	130	129
Standard deviation	44	23	18	5
Poisson estimate of zero amplifiable molecules per well	1090	1184	1150	1151
Poisson estimate of one amplifiable molecule per well	175	92	123	122
Poisson estimate of two amplifiable molecules per well	14	4	7	6
Poisson estimate of three amplifiable molecules per well	1	0	0	0
Poisson estimate of average amplifiable molecules per well	0.16	0.078	0.11	0.11

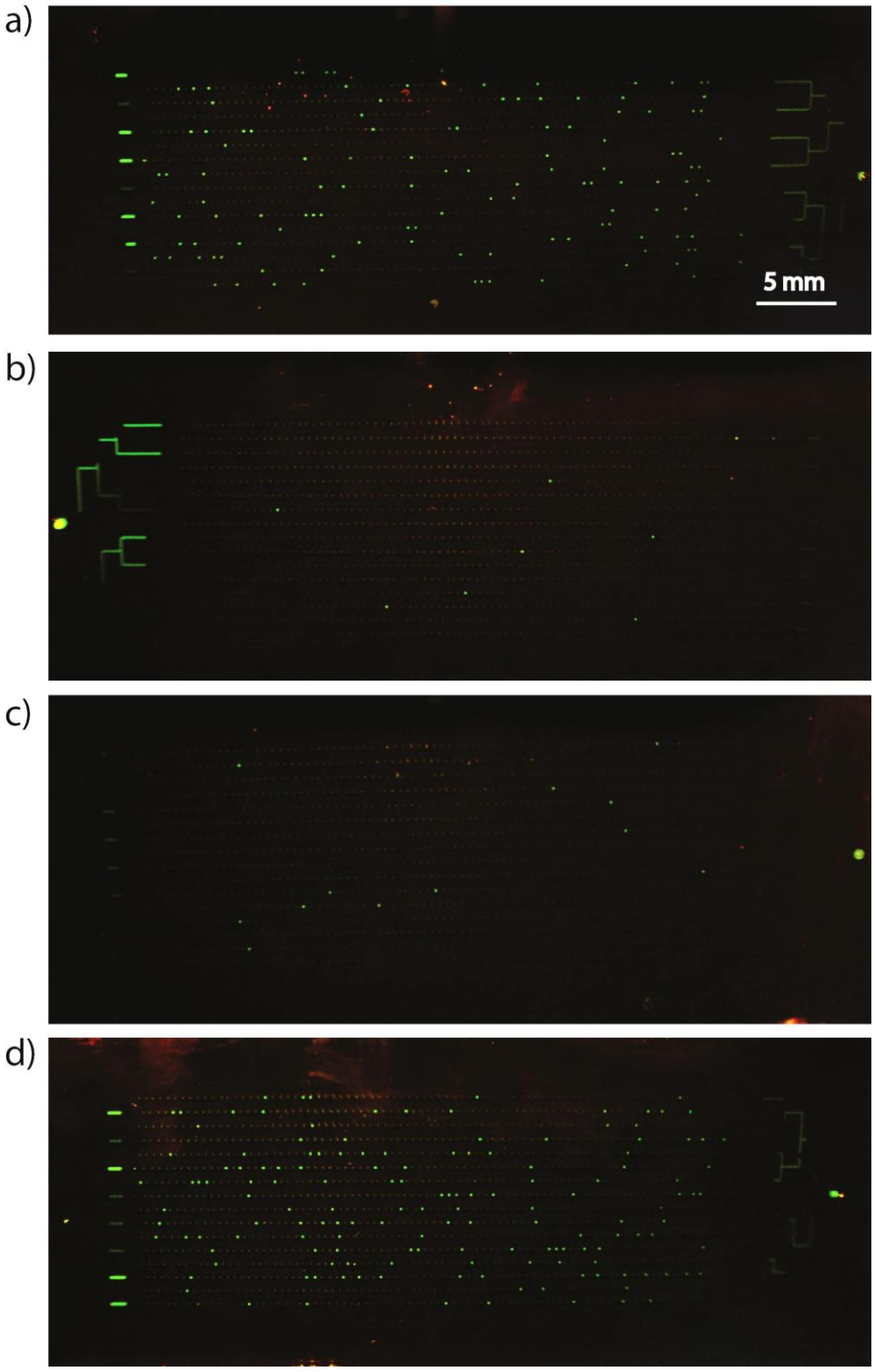

Figure S6 Images of end-point digital RT-LAMP/RE experiments acquired by a cell phone. Positive wells were clearly distinguished from negative wells, indicating the compatibility of this assay with cell phone imaging. The combinations of RNA genotypes and REs are: a) GT3 without RE; b) GT3 with BsrBI; c) GT1 with BsrBI; and d) GT3 with BstNI.

References

[1] B. Sun, F. Shen, S. E. McCalla, J. E. Kreutz, M. A. Karymov, R. F. Ismagilov, *Anal. Chem.* **2013**, *85*, 1540-1546.

[2] a) W. Du, L. Li, K. P. Nichols, R. F. Ismagilov, *Lab Chip* **2009**, *9*, 2286-2292; b) F. Shen, B. Sun, J. E. Kreutz, E. K. Davydova, W. Du, P. L. Reddy, L. J. Joseph, R. F. Ismagilov, *J. Am. Chem. Soc.* **2011**, *133*, 17705-17712.

[3] D. A. Selck, M. A. Karymov, B. Sun, R. F. Ismagilov, *Anal. Chem.* **2013**, *85*, 11129-11136.