**SUPPORTING INFORMATION**

# Multiplexed Droplet Loop-mediated Isothermal Amplification with Scorpion-shaped Probes and Fluorescence Microscopic Counting for Digital Quantification of Virus RNAs

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## ADDITIONAL EXPERIMENTAL SECTION

**Chemicals and Materials.** TE buffer (pH 8.0), Tris-borate-EDTA (TBE) buffer, DNA Marker (100-5000 bp), bovine serum albumin (BSA), RNase-free water, All-In-One DNA/RNA Mini-Preps Kit and DNA oligonucleotides used in this study were purchased from Sangon Biotech (Shanghai, China), and purified by HPLC. The sequences of DNA were given in Table S1. Bst 2.0 WarmStart™ DNA polymerase, deoxyribonucleotides (dNTPs) mixture, $MgSO_4$, Thermopol buffer and DNase I (RNase-Free) were obtained from New England Biolabs Inc. (Ipswich, MA, USA). 5% Pico-Surf™ 1 in Novec-7500 was bought from The Dolomite Centre Ltd. (Anglian Business Park, Royston, UK). Fluorescein sodium and octadecyltrichlorosilane were purchased from Sigma Aldrich (St. Louis, Mo, USA). AZ1805 photoresist coating, Type SG3006 glass substrate with chromium and glass cover plates were bought from Shaoguang Microeletronics Corp (Changsha, China). QIAamp MinElute Virus and QuantiTect Reverse Transcription Kit were purchased from QIAGEN (Hilden, Germany). The clinic HCV and HIV samples were provided by The Third Xiangya Hospital of Central South University (Changsha, China), and the nucleic acids of HCV and HIV were extracted by QIAamp MinElute Virus Spin Kit. HCV and HIV cDNA were obtained by reverse transcription according to the instruction of QuantiTect Reverse Transcription Kit. Ultrapure water was used for preparing all solutions, obtained through a Millipore water purification system (Billerica, MA, USA) with an electric resistance > 18.25 MΩ. All chemicals were analytical grade unless otherwise indicated.

**Instruments.** The Real-time fluorescence intensity study was performed using a C1000 Thermal Cycler (Bio-Rad, CA, USA) with a CFX96. Agarose gel was visualized using a Tanon 4200SF Gel Imaging System (Tanon Science & Technology, Shanghai, China). Aqueous and oil phase were injected into the microfluidic device by using TS-2A Syringe Pump System (Longer Precision Pump, Hebei, China) with 100 μL syringe (Hamilton, Reno, NV). Photomask pattern was transferred onto SU-8 photoresist via URE-2000/25 ultraviolet photolithographic machine (Institute of Optics and Electronics, Chinese Academy of Sciences, Sichuan, China). All fluorescence images were acquired using an objective (10×) on Nikon TI-E+A1 SI confocal laser scanning microscope (Nikon, Tokyo, Japan).

**Standard multiplexed LAMP reaction.** DNA targets were heated at 95 °C for 5 min and then snap-cooled on the mixture of ice and water for at least 20 min before use. The multiplexed LAMP reaction was carried out in a volume of 25 μL containing 1× ThermoPol buffer (10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 10 mM KCl, 0.1% Triton X-100, pH 8.8), 1.6 μM HCV and HIV

internal primers FIP, 1.6 μM HCV and HIV internal primers BIP, 0.2 μM HCV and HIV outer primers F3, 0.2 μM HCV and HIV outer primers B3, 0.4 μM HCV and HIV loop primers LF, 0.4 μM HCV and HIV scorpion-shaped probes SP, 1.6 mM dNTPs, 6 mM $MgSO_4$, 0.32 U/μL Bst 2.0 WarmStart™ DNA polymerase and different concentrations of target DNA or extracted cDNA. The reaction was incubated at 64 °C for 1 h. The real time fluorescence intensity was monitored at 64 °C on a C1000 Thermal Cycler with a CFX96 detection system at intervals of 30 s for 100 cycles using the ROX channel and Cy5 channel simultaneously. Then we heated the reaction products to 80 °C for 20 min to denature the polymerase for the further analysis.

**Agarose gel electrophoresis analysis.** The products of LAMP reaction were used for 3% agarose gel electrophoresis with adding 10 μL of each sample into the lanes. The agarose gel was stained with 0.5 μg/mL Goldview and 0.5 μg/mL ethidium bromide. The electrophoresis was carried out in 0.5× TBE buffer (90 mM Tris-HCl, 90 mM boric acid, and 2 mM EDTA, pH 8.0) at a constant voltage of 90 V for 1.5 h at room temperature. The images of the gel were visualized with Tanon 4200SF gel imaging system.

**Analysis of plasma HCV and HIV RNA.** Sixteen clinical samples were collected from The Third Xiangya Hospital of Central South University. The 2 mL blood plasma samples were separated from the cellular fraction by centrifugation. HCV and HIV RNA was extracted using QIAamp MinElute Virus Spin Kit following the manufacturer's protocol. HCV and HIV cDNAs was obtained by reverse transcription according to the instruction of QuantiTect Reverse Transcription Kit. The cDNA samples were dissolved in 2 mL water and then divided into two equal-volume portions. One portion was subjected to qPCR analysis, and the other was subjected to analysis using the multiplexed dLAMP assay.

**Quantification of HCV cDNA.** A fluorescence quantitative PCR assay was used to quantify HCV cDNA. The forward and reverse primers for qPCR assay are 5'- CACTCGCAAGCACCCTATCA -3' and 5'- AGCCATAGTGGTCTGCGGA -3', respectively. The qPCR experiment was performed in 20 μL reaction mixture containing 10 μL 2× SybrGreen qPCR Master Mix, 2 μL synthetic template or HCV cDNA and 0.4 μL of 10 μM forward and reverse primers on an ABI StepOnePlus qPCR instrument. Cycling conditions were as follows: 3 min at 95 °C followed by 45 cycles of 5 s at 95 °C and 30 s at 60 °C. The concentration of HCV cDNA was determined using a standard curve for the synthetic template.

**Quantification of HIV cDNA.** A fluorescence quantitative PCR assay was used to quantify HIV cDNA. The forward and reverse primers for qPCR assay are 5'- GTAGTTCCTGCTATGTCACTTCCC -3' and 5'- CATTATCAGAAGGAGCCACCC -3', respectively. The qPCR experiment was performed in 20 μL reaction mixture containing 0.4 μL of 10 μM forward and reverse primers, 10 μL 2× SybrGreen qPCR Master Mix and 2 μL synthetic template or HIV cDNA on an ABI StepOnePlus qPCR instrument. Cycling conditions were as follows: 3 min at 95 °C followed by 45 cycles of 5 s at 95 °C and 30 s at 60 °C. The concentration of HIV cDNA was determined using a standard curve for the synthetic template.

**Microfluidic Chip Fabrication.** Using AutoCAD software to draw the Y-shape droplet microfluidic chip pattern and printed it on the dark field mask film. Lithographic transfer of the microchannels on the mask to the glass surface and etching to produce the channel cross section at junction 55 μm (w) × 50 μm (d) and wide channel cross-section 250 μm (w) × 50 μm (d), in the stirred tank filled with diluted $HF/NH_4F/HNO_3$ solution. Use a mechanical drill to etch five 1.8 mm diameter holes at the end of the channel through etching glass. The etched glass was thermally bonded in a muffle furnace at 580 °C for 2 hours to reach a featureless glass substrate and same thickness. After bonding, wash the microchannels with isopropyl alcohol, acetone, piranha solution ($H_2SO_4/H_2O_2$, 3:1) and deionized water in turn, and then dry with nitrogen. At the end, we added the cover, which was bonded to the glass plate by a room temperature bonding process. Following that, glass channels were hydrophobically treated with a 0.1% solution of octadecyltrichlorosilane in dry toluene for 5 min. Wash the treated channel with dry toluene, isopropanol and deionized water in sequence, and dried at 150 °C for 2 h. The chip was then used in the experiments.

The droplet counting microwell chip was designed to collect droplets and the pattern was designed by AutoCAD software. The microchip capture well with hydrophilic channel was fabricated using standard multistep photolithography and wet chemical etching technique based on 1.6 mm-thick 20 × 60 mm borosilicate glass substrates with chromium film. The chip consisted of a main channel (54.7 mm length, 6.4 mm wide and 50 μm deep), an inlet and an outlet. After UV exposure, the photomask pattern was transferred to the glass plate. In a fully stirred bath containing diluted $HF/NH_4F/HNO_3$, the microchannels are etched in the glass substrate with an etching depth of 50μm. Then, we used the previous chromium etchant solution to completely remove the remaining chromium layer. And the glass plate was ultrasonic cleaned with acetone, household washing powder, ultrapure water and ethanol in turn, dried in an oven at 150 °C for 2 h. In the end, we added

the cover, which was bonded to the glass plate through a room-temperature bonding process. Finally, the whole surface of the droplet counting microwell chip was subjected to silanization with 1% octadecyltrichlorosilane in isooctane (v/v) to acquire hydrophobic surface. After cleaning and drying, we fabricated a droplet counting microwell chip surrounded by a hydrophobic coating. These microchips can be used repeatedly.

**Multiplexed dLAMP assay.** The oil phases consisted of 1% Pico-Surf™ 1 in fluorinated oil Novec-7500. One of the water phases comprised 2× ThermoPol buffer, 3.2 μM HCV and HIV internal primers FIP, 3.2 μM HCV and HIV internal primers BIP, 0.4 μM HCV and HIV outer primers F3, 0.4 μM HCV and HIV outer primers B3, 0.8 μM HCV and HIV loop primers LF, 0.8 μM HCV and HIV scorpion-shaped probes SP, 3.2 mM dNTPs, 12 mM $MgSO_4$, 0.2 mg/mL BSA, 1 μM fluorescein sodium, 0.64 U/μL Bst 2.0 WarmStart™ DNA polymerase. And the other water phases maintained different concentrations of target DNA or extracted cDNA. Both water phases were mixed on droplet chip. The flow rates were set to 10 μL/min for oil phases and 3 μL/min for water phases during droplet generation. We were used Y-shape droplet microfluidic chip to generate droplets and collected them in droplet counting microwell chip. Then, the multiplexed dLAMP reaction was performed at 64 °C for 60 min. After observation, we added isopropanol to clean the chip and incubated 5 U/mL DNase I at 37 °C for overnight to degrade residual oligonucleotides.

**Confocal Fluorescence Imaging.** We incubated droplet counting microwell chip at 64 °C for 60 min and acquired fluorescence images by using an inverted confocal laser scanning microscope under 10× objective. We used 488 nm, 560 nm and 640 nm laser as the excitation source simultaneously. Then, we analyzed the pictures and calculated different positive droplets with NIS-Elements software and then counted the template copies according to the Poisson distribution.

**Ethical clearance.** All of patient samples were provided by The Third Xiangya Hospital of Central South University. And the permission to use the patient samples was obtained from the ethical committee of The Third Xiangya Hospital of Central South University. Viral RNA purification and reverse transcription with clinical samples were strictly performed in Biosafety Level 2 laboratory. For safety, the HCV and HIV cDNA were inactivated by heating and preserved for further analysis.

**Table S1.** Synthesized DNA primers and templates for multiplexed dLAMP analysis.

| Name | Sequence (5'-3') |
|---|---|
| HCV-F3 | TGGTCTGCGGAACCGG |
| HCV-B3 | GGGGCACTCGCAAGCA |
| HCV-FIP | ACGCCCAAATCTCCAGGCATTGCATTGCCAGGACGACCGG |
| HCV-BIP | CCGCGAGACTGCTAGCCGACCCTATCAGGCAGTA |
| HCV-LF | AGCGGGTTGATCCAAGAAAGGAC |
| HCV-LB | TGTTGGGTCGCGAAAGGCC |
| HCV-SP | TAMRA-AGCGCGGATATCTCACCGCGCT(BHQ2)TGTTGGGTCGCGAAAGGCC |
| HCV template | GCCAGCCCCCTGATGGGGGCGACACTCCACCATGAATCACTCCCCT<br>GTGAGGAACTACTGTCTTCACGCAGAAAGCGTCTAGCCATGGCGT<br>TAGTATGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGA<br>GCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGAATTGCCAGG<br>ACGACCGGGTCCTTTCTTGGATCAACCCGCTCAATGCCTGGAGATT<br>TGGGCGTGCCCCCGCGAGACTGCTAGCCGAGTAGTGTTGGGTCGC<br>GAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCC<br>GGGAGGTCTCGTAGACCGTGCACCATGAGCACGAATCCTAAAC |
| HIV-F3 | CCTATTTGTTCCTGAAGGGT |
| HIV-B3 | ATTATCAGAAGGAGCCACC |
| HIV-FIP | GAGTGCATCCAGTGCATGCACTGCTATGTCACTTCCCCT |
| HIV-BIP | CCATTCTGCAGCTTCCTCATTGAACACCATGCTAAACACAGT |
| HIV-LF | CAGGCCAGATGAGAGAACCA |
| HIV-LB | ATGGCTGCTTGATGTCCCC |
| HIV-SP | Cy5-AGCGCGGATATCTCACCGCGCT(BHQ2)ATGGCTGCTTGATGTCCCC |
| HIV template | ATTTTATTTAATCCCAGGATTATCCATCTTTTATAAATTTCTCCTAC<br>TGGGATAGGTGGATTATTTGTCATCCATCCTATTTGTTCCTGAAGG<br>GTACTAGTAGTTCCTGCTATGTCACTTCCCCTTGGTTCTCTCATCTG<br>GCCTGGTGCAATAGGCCCTGCATGCACTGGATGCACTCTATCCCAT<br>TCTGCAGCTTCCTCATTGATGGTCTCTTTTAACATTTGCATGGCTGC<br>TTGATGTCCCCCCACTGTGTTTAGCATGGTGTTTAAATCTTGTGGG<br>GTGGCTCCTTCTGATAATGCTGAAAACATGGGTATCACTTCTGGGC<br>TGAAAGCCTTCTCTTCTACTACTTTTACCCATGCATTTAAAGTTCTA<br>GGTGATATGGCCTGATGTACCA |

In designing the primers for LAMP assays, specificity analysis was performed through BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the specific sequences for the pair of viruses of interest.

**Table S2.** Quantification results of serial diluted cDNA by multiplexed dLAMP for HCV and HIV detection.

| Target | | 12 copies | 24 copies | 120 copies | 240 copies | 1200 copies | 2400 copies |
|---|---|---|---|---|---|---|---|
| HCV | Mean | 12.13 | 23.63 | 120.00 | 236.01 | 1169.79 | 2365.37 |
| | Standard deviation | 2.01 | 0.65 | 5.79 | 27.58 | 78.52 | 205.17 |
| | Coefficient of variation (%) | 16.57 | 2.75 | 4.83 | 11.69 | 6.71 | 8.67 |
| | Relative errors (%) | 1.08 | -1.54 | 0.00 | -1.66 | -2.51 | -1.44 |
| HIV | Mean | 12.04 | 23.38 | 118.41 | 233.28 | 1175.69 | 2349.54 |
| | Standard deviation | 1.41 | 1.31 | 10.62 | 15.31 | 22.10 | 52.47 |
| | Coefficient of variation (%) | 11.71 | 5.60 | 8.97 | 6.56 | 1.88 | 2.23 |
| | Relative errors (%) | 0.33 | -2.58 | -1.33 | -2.80 | -2.03 | -2.10 |

Mean, standard deviation (SD), coefficient of variation (CV) and relative errors were calculated by four repetitive experiments.

**Table S3.** The calculation of the limit of detection for multiplexed dLAMP.

| | Total droplet number | Fluorescence droplet number |
|---|---|---|
| Blank 1 | 62457 | 1 |
| Blank 2 | 60846 | 2 |
| Blank 3 | 62351 | 2 |
| Blank 4 | 61640 | 3 |
| Mean | 61823.5 | 2 |
| SD | 645.9 | 0.71 |

The counts obtained in four repetitive assays for the blank samples were 1, 2, 2 and 3 with an average count of 2 and a standard deviation (SD) of 0.7, so the limit of detection was estimated to be 4 according to triple SD over the average count.

**Table S4.** Results of clinical samples obtained using qPCR and multiplexed dLAMP.

| Sample | qPCR | Multiplexed dLAMP | Relative errors | Results |
|---|---|---|---|---|
| 1 | 224 | 213 | -4.91% | HCV positive |
| 2 | 1069 | 1048 | -1.96% | HCV positive |
| 3 | 2353 | 2389 | 1.53% | HCV positive |
| 4 | 1159 | 1102 | -4.92% | HCV positive |
| 5 | 129 | 118 | -8.53% | HCV positive |
| 6 | 195 | 183 | -6.15% | HCV positive |
| 7 | 112 | 106 | -5.36% | HCV positive |
| 8 | 9 | 8 | -11.11% | Negative |
| 9 | 5 | 4 | -20% | Negative |
| 10 | 1715 | 1689 | -1.52% | HIV positive |
| 11 | 494 | 488 | -1.21% | HIV positive |
| 12 | 1320 | 1301 | -1.44% | HIV positive |
| 13 | 2238 | 2294 | 2.5% | HIV positive |
| 14 | 1807 | 1784 | -1.27% | HIV positive |
| 15 | 350 | 341 | -2.57% | HIV positive |
| 16 | 1039 | 993 | -4.43% | HIV positive |

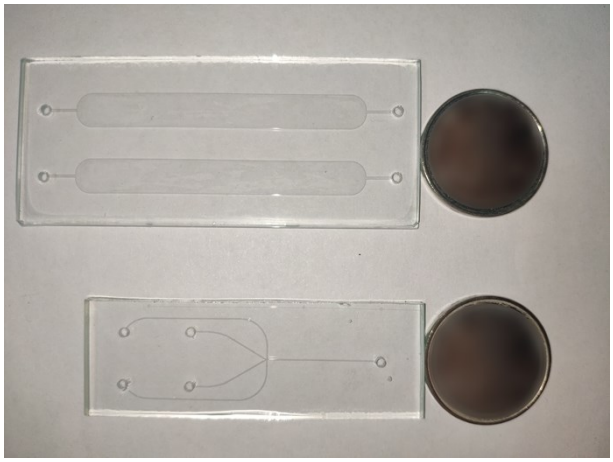

**Figure S1.** Image of droplet counting microwell chip and Y-shape droplet microfluidic chip. The diameter of the coin is 25 mm.

The design of 2 inlets for the oil phase in the Y-shape chip has advantages that the symmetric design counteracts possible physical perturbations in the oil inlets, affording better control of droplet size and droplet frequency.[1]

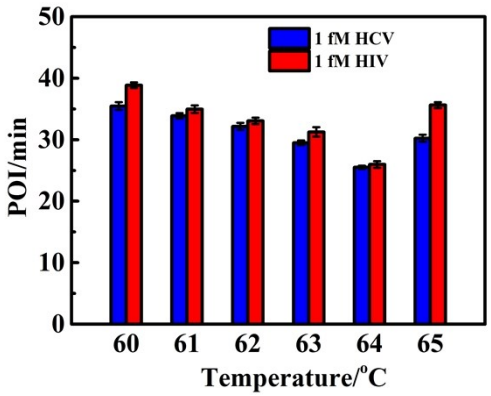


**Figure S2.** Optimization of reaction temperature for the multiplexed LAMP assay. The activity of DNA polymerase depended on the reaction temperature. Therefore, we inspected the incubating temperatures of multiplexed LAMP reaction to achieve high efficiency in multiplex detection. We found the reaction efficiency decreased with temperatures lower or higher than 64 $^{o}$C. Errors bars are standard deviations across three repetitive assays. Therefore, we chose 64 $^{o}$C as the optimized temperature for the following multiplexed LAMP reactions.

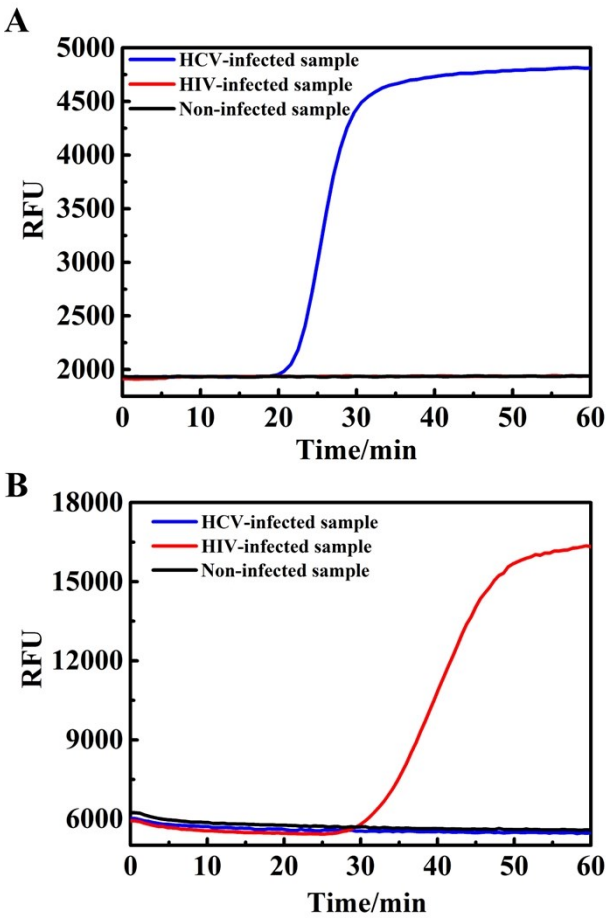


**Figure S3.** Real-time fluorescence curves of multiplexed LAMP reactions for cDNA reverse transcription from extracted RNA of three plasma samples for TAMAR (A) and Cy5 (B), respectively.

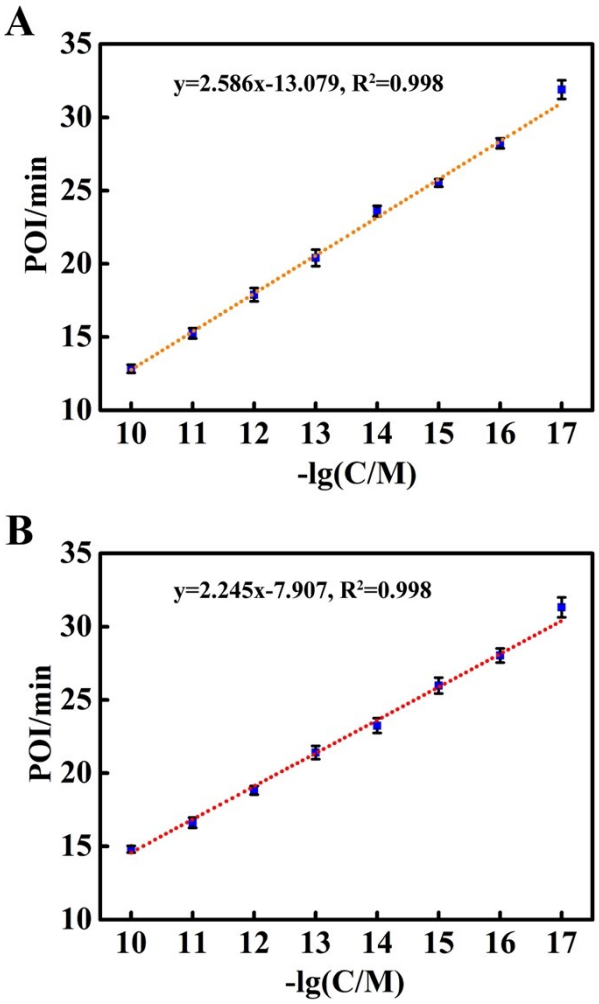


**Figure S4.** Quantitative detection of HCV cDNA (A) and HIV cDNA (B) using multiplexed LAMP assays. Error bars are standard deviations of four repetitive experiments.

To obtain extremely low concentration, serial dilution (10-fold) was performed by adding 100 μL of the concentrated standard solution in 900 μL ultrapure water with disposable pipette tips in a biological cleaning room, which minimized the risk of overestimating the LOD.

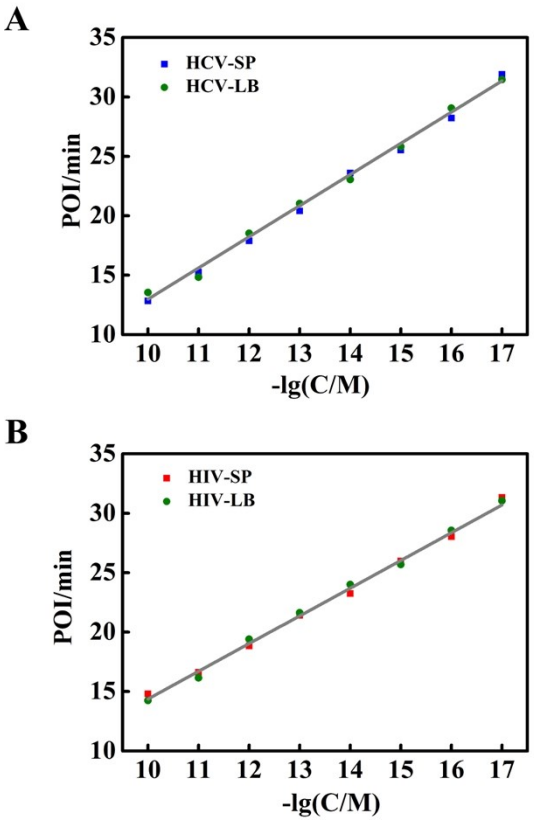


**Figure S5.** Comparison between scorpion-shaped probe (SP) and loop backward primers (LB) for quantitative assays of HCV cDNA (A) and HIV cDNA (B). Concentrations range from $1.0 \times 10^{-17}$ to $1.0 \times 10^{-10}$ M.

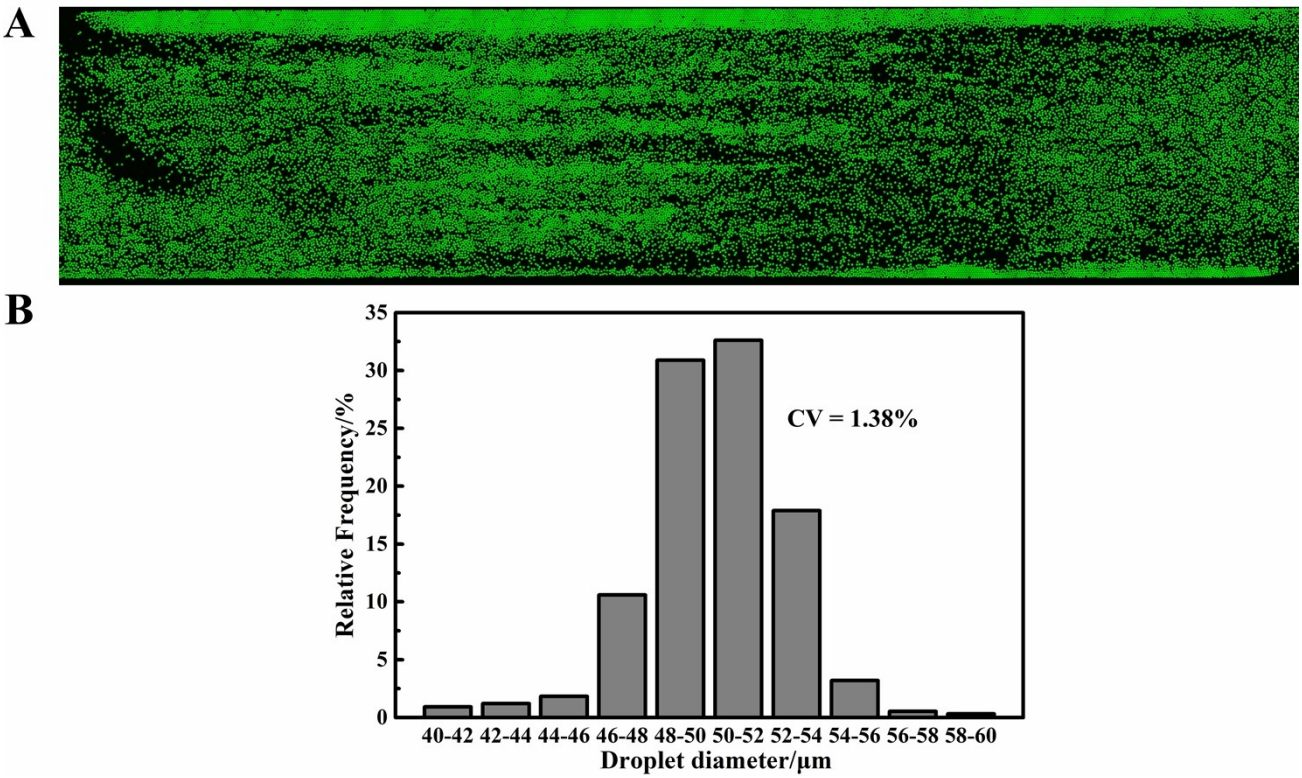


**Figure S6.** The sizes of droplets in whole large microwell image. (A) The fluorescence image of the whole microwell at FITC channel. (B) The diameter distribution and the coefficient of variation about generated droplets.

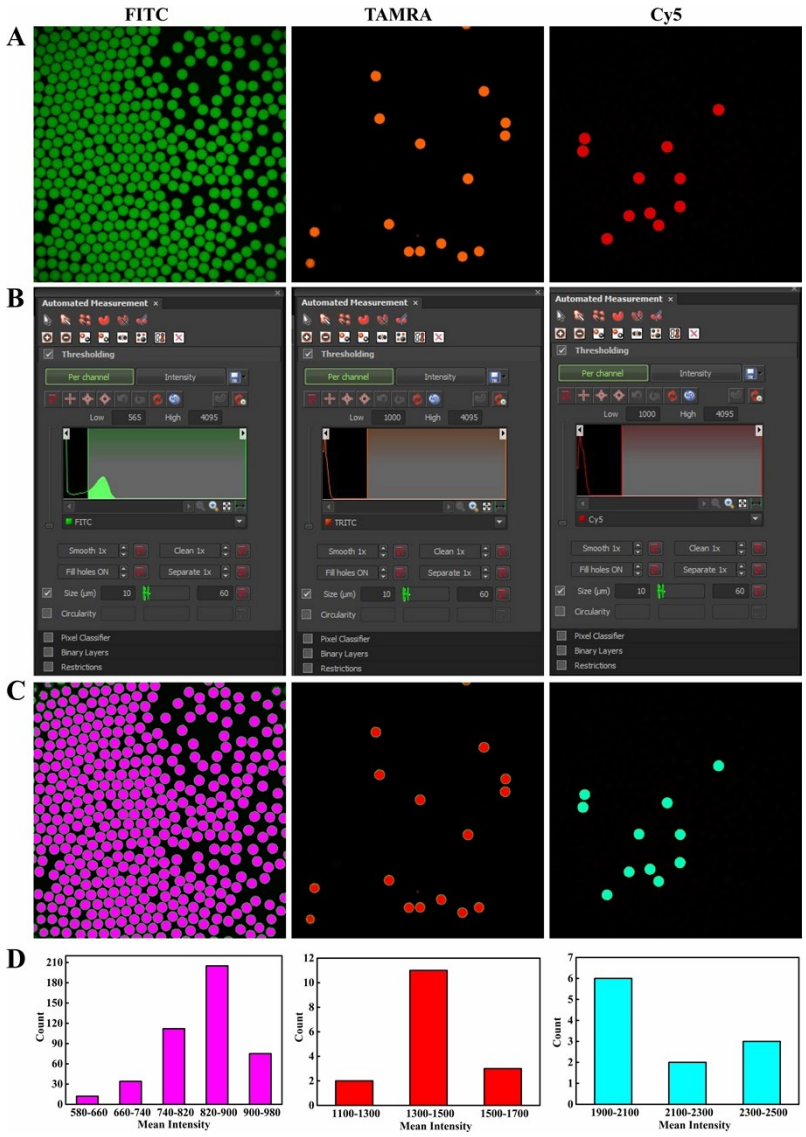


**Figure S7.** Droplet analysis. (A) The fluorescence image of the droplets. (B) The window of Automated Measurement using NIS-Element software. (C) According to a set threshold and size, the color was changed in each fluorescence channel and all droplets were segmented. (D) The number of droplets with Automated Measurement Results.

The process of NIS-Element software to analyze the droplets:

1. Open the NIS-Element software and picture;

2. Click tool bar, choose View → Analysis Controls → Automated Measurement → Thresholding → Per channel, input a proper threshold of the fluorescence intensity and a proper range of droplet size, then run the procedure for analysis;

3. Click tool bar, choose View → Analysis Controls → Automated Measurement Results, then we can get the number of droplets in each fluorescence channel of the pictures.

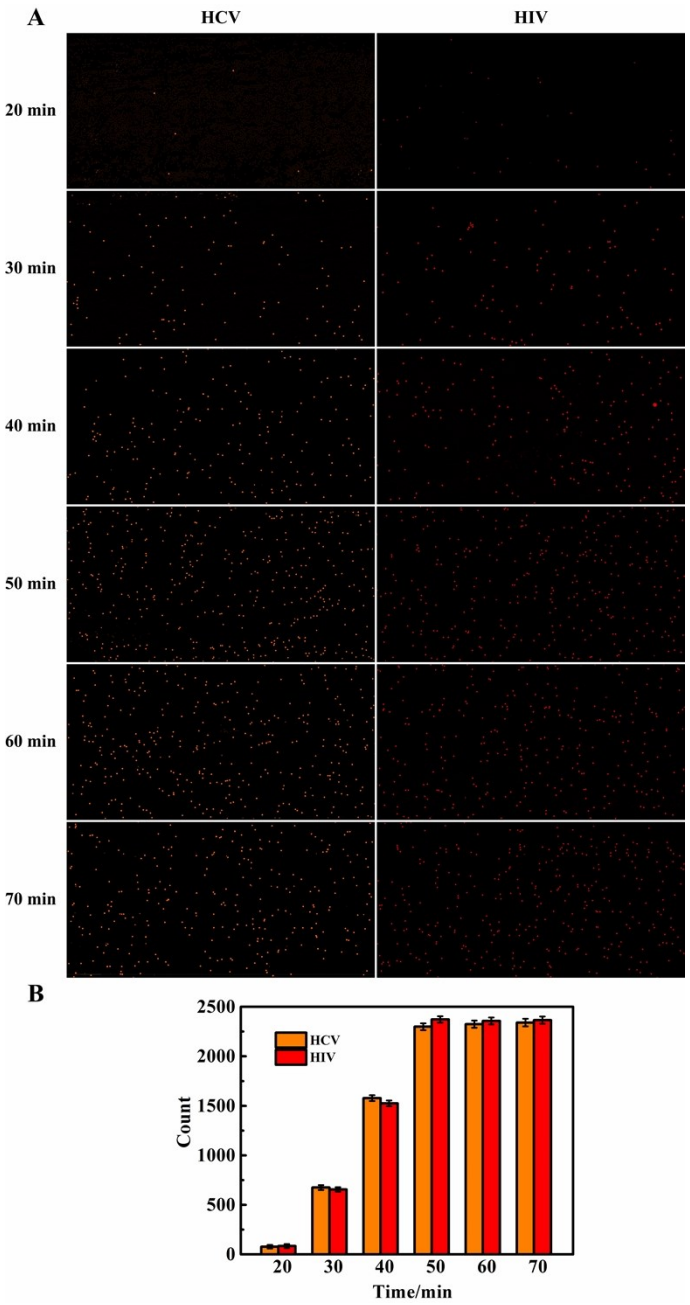


**Figure S8.** Optimization of the reaction time for multiplexed dLAMP. (A) Fluorescence images for effect of reaction time on multiplexed dLAMP. (B) Corresponding counting copy numbers of HCV and HIV cDNA. We optimized the reaction time of multiplexed dLAMP assay for improving its sensitivity. It showed for a reaction time less than 50 min, the detection rates of HCV and HIV were relatively low because the accumulated fluorescence-activated probes were limited. Instead, an overly long reaction time more than 60 min was unable to increase the detection rate. Therefore, 60 min was set as the optimal droplet reaction time for rapid digital assay.

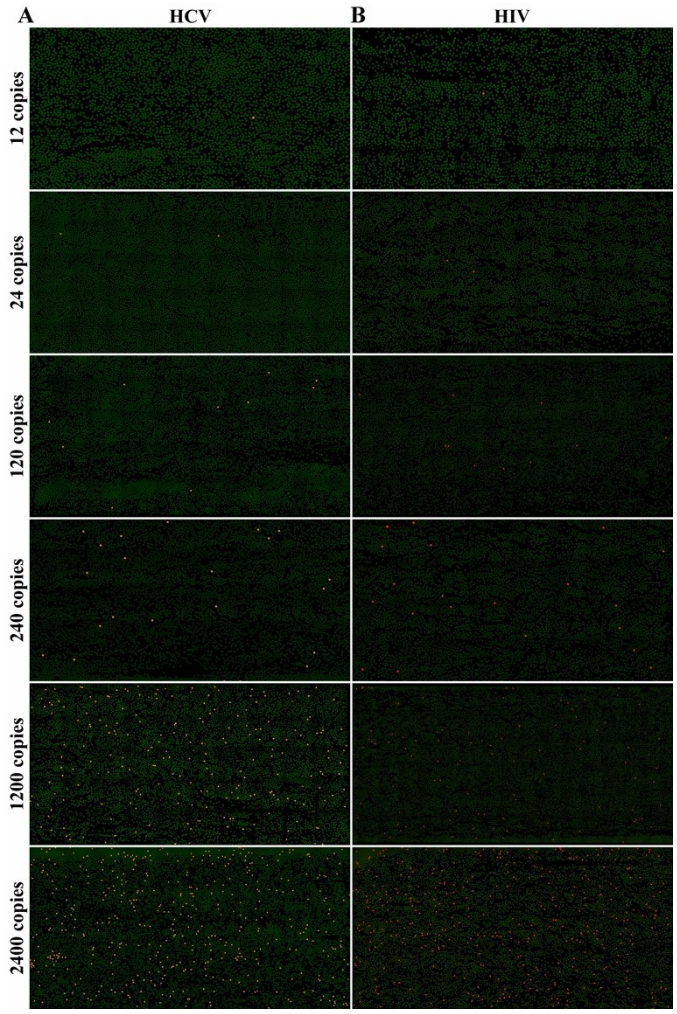


**Figure S9.** The large fluorescence images for quantification of various concentration of HCV and HIV cDNA using multiplexed dLAMP, respectively.

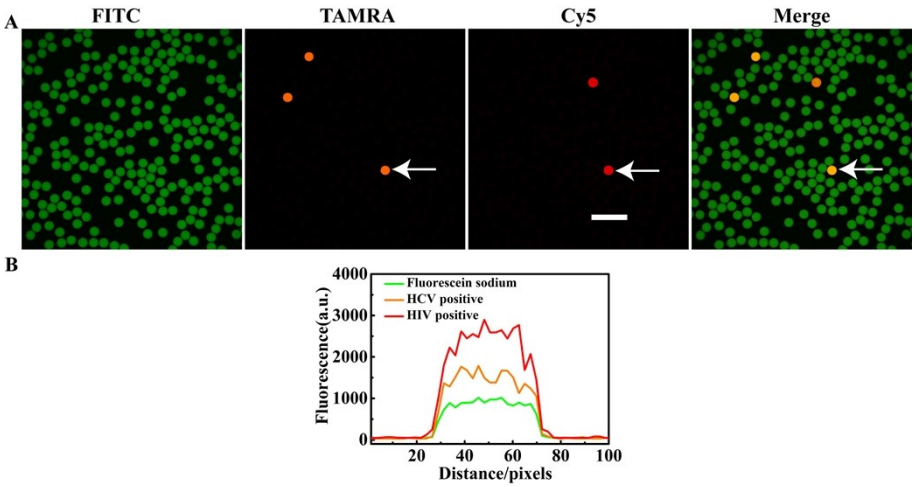


**Figure S10.** Fluorescence images of the droplet containing both HCV and HIV cDNA. (A) Fluorescence images of multiplexed dLAMP reaction of a mixture of each of 1200 copies/μL HCV and 1200 copies/μL HIV cDNA at different channels. Scale bar: 200 μm. (B) Fluorescence profiles of the HCV and HIV positive droplet.

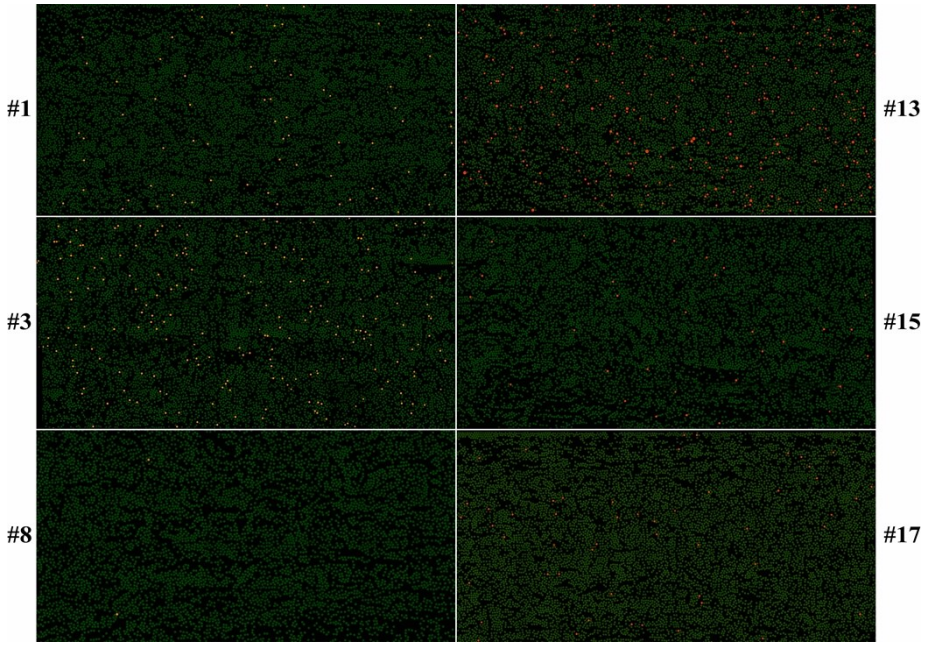


**Figure S11.** Multiplexed dLAMP analysis for six representative clinical plasma samples. Samples 1, 3 and 8 were HCV positive samples; Samples 13, 15 and 17 were HIV positive samples. The estimated HCV and HIV copy numbers of the samples were given in Table S4.

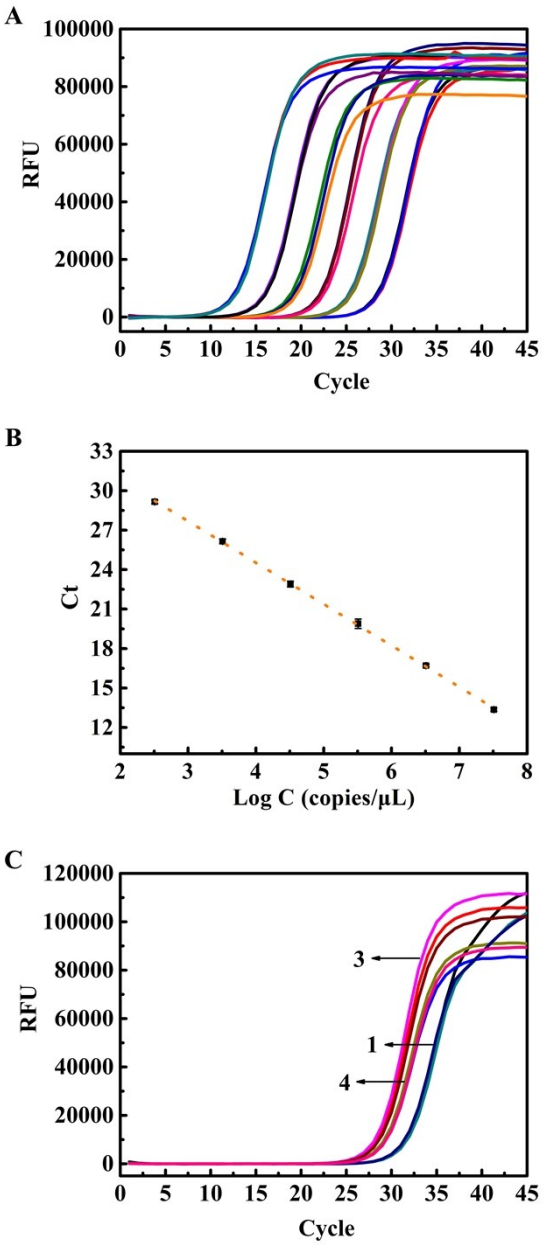


**Figure S12.** qPCR analysis for HCV clinical plasma samples. (A) Real-time fluorescence PCR curves for HCV cDNA of different concentrations (322 ~ 322 × $10^5$ copies/μL). (B) Ct values versus logarithmic cDNA concentrations. (C) Real-time fluorescence PCR curves for three representative clinical samples with 100-fold dilution. The mean Ct values were 31.9, 28.7 and 29.7 for sample 1, 3 and 4, respectively. The estimated HCV copy numbers of the samples were given in Table S4.

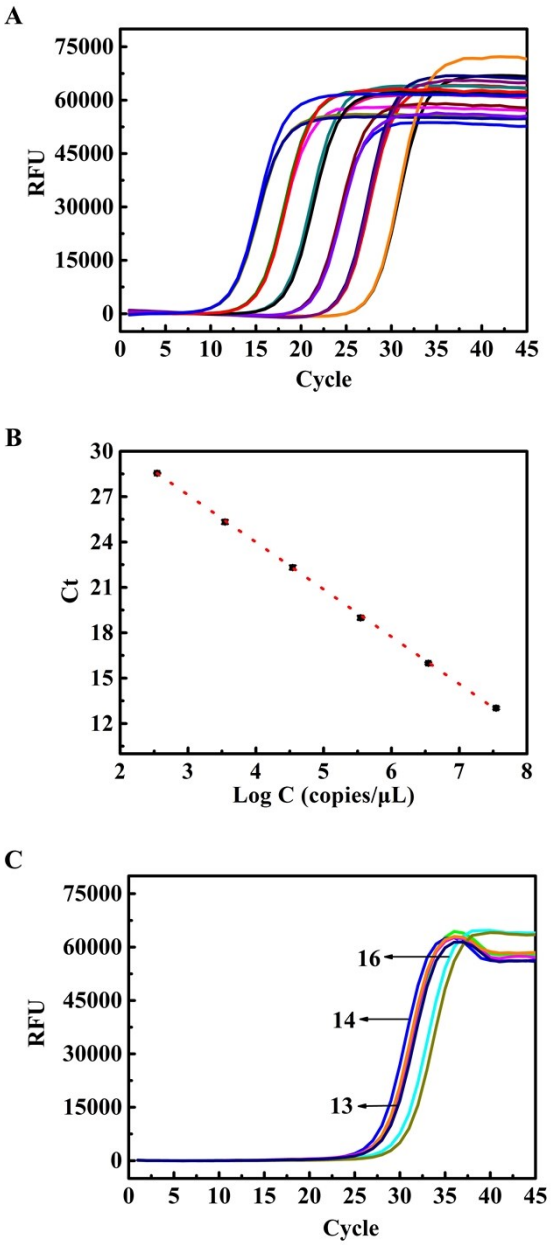


**Figure S13.** qPCR analysis for HIV clinical plasma samples. (A) Real-time fluorescence PCR curves for HIV cDNA of different concentrations (350 ~ 350 × $10^5$ copies/μL). (B) Ct values versus logarithmic cDNA concentrations. (C) Real-time fluorescence PCR curves for three representative clinical samples with 100-fold dilution. The mean Ct values were 28.9, 28.2 and 30.7 for sample 13, 14 and 16, respectively. The estimated HIV copy numbers of the samples are given in Table S4.

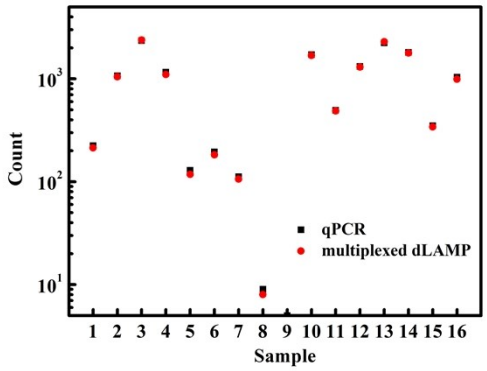


**Figure S14.** Comparison between qPCR and multiplexed dLAMP for quantitation of HCV and HIV viruses in clinical plasma samples.

## References

1. L. Shang, Y. Cheng and Y. Zhao, *Chem. Rev.*, 2017, **117**, 7964-8040.