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

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Experimental section

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™ MF™-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.[1,2] 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 wells of 25 nL and 125 nL for Design 1 (Table 1). For Design 2, this photomask also contained the designs of the additional wells of 625 nL. 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 $HClO_4$ / $(NH_4)_2Ce(NO_3)_6$). 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 wells of 1 nL and 5 nL for Design 1 (Table 1) by using a mask aligner. For Design 2, this second photomask also contained the designs of the additional wells of 0.2 nL. 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 $HF/NH_4F/HNO_3$) 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 (1 nL and 5 nL wells, and the additional wells of 0.2 nL for Design 2). 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 desicator with dichlorodimethylsilane for gas-phase silanization. For Design 2A, circular inlet reservoirs (4 mm inner diameter and 6 mm outer diameter) were made by cutting adhesive PDMS film, then fixing the reservoirs around the five inlets before plasma cleaning. 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) as shown in Figure 1A 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 for Design 1 and Design 2B. The reagent solution was loaded through the inlet by pipetting. For Design 2A, five through-holes were drilled at the top left corner of the top plate to serve as fluid inlets (Figure 5A). 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.[3] 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 µL was mixed with 400 µL lysis buffer (Invitrogen Corporation, Carlsbad, CA) to lyse the virus. Then 2 µL of control RNA (906 nt) was added to characterize the purification efficiency and concentrating factor. The mixed sample was then transferred into the iPrep^{TM} PureLink^{TM} virus cartridge. The cartridge was placed in the iPrep^{TM} purification instrument and the purification protocol was performed

according to the manufacturer's instructions. The final elution volume was 50 μL, 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 (Design 1). The final concentrating factor was 4.5 for HCV and 6.6 for HIV in the multiplex RT-PCR amplification (Figure 5). The concentrating factors for the two HIV samples were 7.1 and 6.6 for the experiments in Figure 6.

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 μL of 2 × EvaGreen SuperMix, 1 μL of each primer (10 μmol/L), 3 μL of BSA solution (20 mg/mL), 1.5 μL of SuperScript® III Reverse Transcriptase, 17.5 μL of nuclease-free water, and 6 μL of template solution.

Primer sequences for HIV viral RNA was selected from a previous publication:[4] 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:[5] 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 in Figure 5, the RT-PCR mix contained the following: 15 μL of 2 × EvaGreen SuperMix, 0.6 μL of each primer (10 μmol/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 in Figure 5, the RT-PCR mix contained the following: 15 µL of 2 × EvaGreen SuperMix, 0.25 µL of each primer (10 µmol/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 Figure 5, the RT-PCR mix contained the following: 15 µL of 2 × EvaGreen SuperMix, 0.25 µL of each primer (10 µmol/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.

The experiment in Figure 5 was repeated six times and all the data was used to calculate the target concentration.

To amplify HIV viral RNA with expected final concentration above 1000 molecules/mL in the RT-PCR mix in Figure 6, the RT-PCR mix contained the following: 20 µL of 2 × EvaGreen SuperMix, 1 µL of each primer (10 µmol/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 serially diluted in 1 mg/mL BSA solution. For experiments with HIV viral RNA concentration below 1000 molecules/mL in the final

RT-PCR mix, the RT-PCR mix contained the following: 30 μL of 2 × EvaGreen SuperMix, 1.5 μL of each primer (10 μmol/L), 2 μL of BSA solution (20 mg/mL), 1.5 μL of SuperScript® III Reverse Transcriptase, 3.5 μL of nuclease-free water, and 20 μL of template solution.

To amplify the control RNA (906 nt) in the HIV sample in Figure 5 and Figure 6, the RT-PCR mix contained the following: 20 μL of 2 × EvaGreen SuperMix, 1 μL of each primer (10 μmol/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 on SlipChip Design 1 with the RT-PCR mix contained the following: 20 μL of 2 × EvaGreen SuperMix, 1 μL of each primer (10 μmol/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.

To amplify HIV viral RNA in Figure S1, the RT-PCR mix for HIV viral RNA contained the following: 90 μL of 2 × EvaGreen SuperMix, 3.6 μL of each primer (10 μmol/L), 6 μL of BSA solution (20 mg/mL), 4.5 μL of SuperScript® III Reverse Transcriptase, 12.3 μL of nuclease-free water, and 60 μL of template solution. The template solution used here was diluted 62500-fold from the original HIV viral RNA stock solution purified from Patient sample 2 using 1 mg/mL BSA solution. This experiment was repeated six times and all data was used to calculate HIV viral RNA concentration. Three negative control experiments

were performed with the same primer pairs but no HIV viral RNA, and showed no false positives.

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 except for those in Figure S1, where 39 cycles were adapted instead of 35 cycles.

Image acquisition and analysis

Bright-field images in Figure 1 and Figure 5 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).

References:

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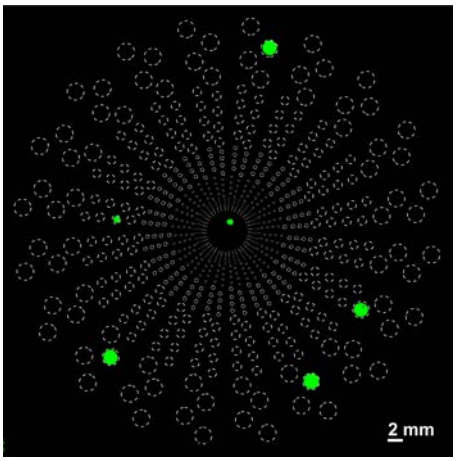

Figure S1. Representative experiment performing RT-PCR of HIV viral RNA at an expected concentration of 51 molecules/mL in RT-PCR mix on the Design 2B SlipChip to test the lower detection limit of the device. This experiment was repeated six times to quantify the viral RNA concentration.

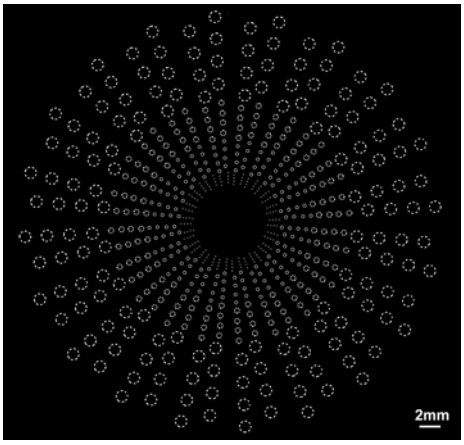

Figure S2. Representative negative control for HIV viral load (HIV primers with no loaded HIV RNA template) on SlipChip Design 1, corresponding to experiments shown in Figure 6.

Table S1. Performance of quantification of HIV viral RNA concentration from patient 1 on SlipChip comparing to Roche COBAS® AmpliPrep / COBAS® TaqMan® HIV-1 Test, v2.0 system (CAP/CTM v2.0). Each experiment was repeated at least four times on SlipChip. Only 2 significant digits are shown. The expected HIV concentration of patient plasma was calculated based on dilution factors and a single result from Roche CAP/CTM v2.0. The results from SlipChip are obtained with serial diluted purified patient HIV viral RNA and are converted to the original concentration in patient plasma (with or without dilutions) using the purification concentrating factor.

Dilution Factor	Expected p HIV viral RNA concentration based on a Roche CAP/CTM v2.0 test (molecules/mL)	Average of calculated HIV viral RNA concentration based on experiments on SlipChip (molecules/mL)	Standard deviation of calculated HIV viral RNA concentration based on experiments on SlipChip (molecules/mL)
1	2.3×10^5	1.6×10^5	1.3×10^4
1:10	2.3×10^4	1.3×10^4	3.0×10^3
1:100	2.3×10^3	1.4×10^3	6.5×10^2
1:1000	2.3×10^2	93	32

Table S2. Performance of quantification of HIV viral RNA concentration from patient 2 on SlipChip comparing to Roche COBAS® AmpliPrep / COBAS® TaqMan® HIV-1 Test, v2.0 system (CAP/CTM v2.0). Each experiment was repeated at least four times on SlipChip. Only 2 significant digits are shown. The expected HIV concentration of patient plasma was calculated based on dilution factors and a single result from Roche CAP/CTM v2.0. The results from SlipChip are obtained with serial diluted purified patient HIV viral RNA and are converted to the original concentration in patient plasma (with or without dilutions) using the purification concentrating factor.

Dilution Factor	Expected p HIV viral RNA concentration based on a Roche CAP/CTM v2.0 test (molecules/mL)	Average of calculated HIV viral RNA concentration based on experiments on SlipChip (molecules/mL)	Standard deviation of calculated HIV viral RNA concentration based on experiments on SlipChip (molecules/mL)
1	1.5×10^6	1.7×10^6	3.9×10^5
1:20	7.3×10^4	9.2×10^4	4.0×10^4
1:400	3.6×10^3	3.3×10^3	6.3×10^2
1:8000	1.8×10^2	1.8×10^2	81
1:40000	36	37	37

Complete Reference 15:
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Author Contributions:
F.S., J.E.K., B.S., W.D. and R.F.I. designed the SlipChip experiments. 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.