Supporting Information

**Title:** Multistep SlipChip for the Generation of Serial Dilution Nanoliter Arrays and Hepatitis B Viral Load Quantification by Digital Loop Mediated Isothermal Amplification

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METHODS

Chemicals and materials

All solvents and salts obtained from commercial sources were used as received unless otherwise stated. Perchloric acid, ammonium fluoride, hydrogen peroxide, sulfuric acid, nitric acid, chloroform, acetone, and ethanol were purchased from SinoPharm Chemical Reagent Co., Ltd (Beijing, China). Dichlorodimethylsilane, tetradecane, and hydrofluoric acid were purchased from Innochem Technology Co., Ltd (Beijing, China). Ammonium ceric nitrate was obtained from Macklin Biochemical Co., Ltd (Shanghai, China). Spectrum food colors were obtained from August Thomsen Corp (Glen Cove, NY). Soda-lime glass plates coated with chromium and photoresist was purchased from Telic Company (Valencia, CA) and photomasks were obtained from MicroCAD Photo-Mask LTD (Shenzhen, China). Oligo nucleotides were synthesized by Sangon Biotech, (Shanghai, China). Bst 2.0 WarmStart™ DNA polymerase and dNTPs were purchased from New England Biolabs (Beijing, China). EvaGreen was obtained from Biotium (Shanghai, China). BSA solution, propidium iodide staining kit, Fluorescence was obtained from Sangon Biotech.

Fabrication of the SlipChip device

The SlipChip microfluidic plates were fabricated using a wet etching method from soda-lime glass plates. The glass plate pre-coated with a chromium layer and a photoresist layer was aligned with a film photomask, and exposed to UV light in a UV flood curing system (Uvitron International Inc., West Springfield, MI) for 12 sec. Then, the exposed glass plate was submerged in 0.1 mol/L NaOH solution for 1 minute to remove the reacted photoresist. Next, the glass plate was placed in a chromium etchant solution containing 0.6 mol/L HClO$_4$ and 0.365 mol/L ($\text{NH}_4$)$_2$Ce(NO$_3$)$_6$ to
remove the exposed chromium layer. After thoroughly rinsed with water, the glass plate was then immersed in a glass etchant solution containing 0.5 mol/L NH₄F, 1 mol/L HF, and 0.75 mol/L HNO₃. The glass etching was carried out in a shaking water bath with temperature at approximately 40 °C to have a uniform etching rate. The etching depth was frequently monitored by using a profilometer (Bruker, Billerica, MA). After etching, the glass plate was rinsed with water, and placed in EtOH for 1 minute to remove the remaining photoresist. Then, the glass plate was dried with air and submerged in chromium etchant solution to remove the remaining chromium layer.

The surface of the glass plate was silanized to be hydrophobic using a gas-phase silanization protocol. The glass plate was oxidized in a plasma cleaner for 120 seconds and immediately placed in a desiccator with 25 µL of dichlorodimethylsilane. A vacuum was applied and maintained for approximately 40 minutes, and then the glass plate was thoroughly rinsed with chloroform, acetone, and ethanol.

**Operation of the SlipChip device**

The operation of the SlipChip device was described in detail previously. In brief, the top and bottom layers of the SlipChip device were aligned under a stereoscope (SMZ1270, Nikon, Japan) and assembled together with a lubricant (mineral oil: tetradecane at a 1:1 volumetric ratio) placed in between. The two layers can be fixed by a clumping fixture. Solution can be introduced into the device at the loading position with a simple pipetting step, and then the top layer can be slipped against the bottom layer manually.

**Measurement of mixing between mother and daughter wells**
We first characterized the mixing between mother and daughter microwells by using fluorescein solution. A solution containing 100 ng/ml fluorescein was introduced into mother microwells (12 nL, 50 µm deep, 500 µm diameter) on the top plate; a solution containing dilution buffer was loaded into daughter microwells (12 nL, 50 µm deep, 500 µm diameter) on the bottom plate. The mother microwells were brought into contact with the daughter microwells by relative movement of top and bottom microfluidic plates. After incubation times of 15 seconds, 30 seconds, 1 minute, and 2 minutes, the top plate was slipped relative to the bottom plate to separate mother microwells from daughter microwells. The fluorescence intensity of twenty pairs of microwells was measured by using a Nikon Ti2 fluorescence microscope (Fig. S1A). The results indicated that the mixing of fluorescein by passive diffusion was sufficient at 1 minute or longer incubation times.

Furthermore, we characterized the mixing between mother and daughter microwells by using a DNA template of 5034 bp. The DNA template was cloned from the Tet-pLKO-puro plasmid (Addgene) using the forward primer 5′-GATAGTTACCGGATAAGGCGCAGCG-3′ and the reverse primer 5′- TAGTGAGACGTGCGGCTTCCGTTTG-3′. A solution containing 95% amplified DNA template and 5% propidium iodide (PI) was introduced into the mother microwells (12 nL, 50 µm deep, 500 µm diameter) on the top plate; a solution containing dilution buffer was loaded into daughter microwells (12 nL, 50 µm deep, 500 µm diameter) on the bottom plate. The mother microwells were brought into contact with the daughter microwells by relative movement of top and bottom microfluidic plates. After incubation times of 15 seconds, 30 seconds, 1 minute, 2 minutes and 3 minutes, the top plate was slipped relative to the bottom plate to separate mother microwells from daughter microwells. The fluorescence intensity of twenty pairs of microwells was measured by using a Nikon Ti2 fluorescence microscope (Fig. S1B). The results indicated that the
mixing of PI by passive diffusion was sufficient at 2 minutes.

**Standard panels and clinical samples**

A 713 bp fragment encompassing a partial HBV small S protein region was PCR-amplified from a positive serum using the forward primer 5’-ATGGAGAACATCACATCAGG-3’ and the reverse primer 5’-GGAATAACCCCATCTCTTTG-3’. DNA fragments were cloned into *Escherichia coli* DH5α cells using the PESI-T Vector System. The resulting recombinant plasmids were extracted with the AxyPrep Plasmid Miniprep Kit, and their concentration was determined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). A standard template was diluted by serial 10-fold dilutions with diethyl pyrocarbonate (DEPC)-treated water to prepare template DNA solutions at concentrations ranging from $3.78 \times 10^5$ copies/μL (1 pg/μL) to $3.78 \times 10^{10}$ copies/μL (100 ng/μL). Clinical specimens were obtained from Renji Hospital. Plasma samples of sixteen anonymous patients were used in this study. HBV DNA was first purified and quantified by using an HBV DNA quantification kit (cat no. KM0612, Perkin-Elmer) on an ABI 7500 instrument and on an sd-SlipChip in parallel.

**Calculation of theoretical dynamic range:**

The lower detection limit (LDL) and the upper limit of quantification (ULQ) were calculated based on the algorithm described previously. The average concentration of LDL, $\lambda$, requires three molecules in the total volume. For the eight-step sd-SlipChip in this paper, the mother microwells was 3 nL and the volume of complementary mother microwells was 12 nL. Three molecules in 0.96 μL (3 nL/well×64 wells + 12 nL/well×64 wells) correspond to an average concentration of
approximately 4 copies/μL. The ULQ was calculated based on the number of wells and volume per each well of the 8th row of daughter microwells with the following equation:

$$0.05 = (1 - e^{-(v \cdot \lambda)})^n$$

Where \(v\) was 12 nL and \(n\) was 64, respectively. The ULQ is about \(1.0 \times 10^8\) copies/μL. The theoretical dynamic range is defined as the ULQ/LDL.² The eight-step sd-SlipChip can achieve a quantification dynamic range of \(2.5 \times 10^7\), while the traditional digital NAA requires approximately 5 million of 1nL droplets.

**Calculation of copy number for digital amplification results**

The calculation method of the concentrations was based on maximum-likelihood estimation (MLE) combined with Poisson estimators, and it was described previously for serial dilution PCR in tubes.³ The number of positive wells for each row was counted. For randomly dispersed nucleic acid molecules, molecules are encapsulated according to Poisson statistics. The probability that a well contains \(k\) molecules is therefore given by

$$p = e^{-\lambda} \frac{\lambda^k}{k!}$$

where \(\lambda\) is the average number of molecules per well. For random single-molecule encapsulation, the probability of a negative well is 36.8% when \(\lambda = 1\). In the sd-SlipChip, the first row containing fewer than 40 positive counts (approximately 63.2%) or, in another way, more than 24 negative counts (approximately 36.8%) was considered the base row. The base row and the rows below (more diluted) were inserted into the algorithm to calculate the original concentration of the target nucleic acid.
LAMP reaction

A 192 bp fragment was amplified by the primers as described previously (Table S1). To prepare a 25 μl of LAMP reaction mixture, 0.8 μM each of the forward inner primer (FIP) and the backward inner primer (BIP), 0.1 μM each of the forward outer primer (F3) and the backward outer primer (B3), 0.4 μM each of the forward loop primer (LF) and the backward loop primer (BF), 1 μL of 10× Isothermal Amplification Buffer, 6 mM of MgSO$_4$, 8 U of Bst DNA polymerase, 1.4 mM each of the dNTPs, 1.25 μL of 20 mg ml$^{-1}$ BSA and 1.25 μL of 20× EvaGreen Dye were mixed. 11.4 μL of the template was added to the mixture, and the reaction was conducted at 65 °C for 30 minutes on an *in situ* thermal cycler (BIO-GENER, China).

Analysis of fluorescence intensity for digital LAMP on the sd-SlipChip

The fluorescence intensity of the digital LAMP on the sd-SlipChip device was measured by using a Nikon Ti2 fluorescence microscope. All images of the digital LAMP experiments were processed with the same image analysis protocols. The background was subtracted from the measured intensity. The fluorescence intensity from a no-template control (NTC) experiment (576 wells) after incubation was measured and analyzed. The value of 35 RFU, which was the average fluorescence intensity of NTC plus three standard deviations, was applied as the threshold cut-off value for all digital LAMP experiments.

Measurement of droplet size distribution

LAMP reaction mixture without template was load into an eight-step sd-SlipChip. Then the fluorescence signal was measured with Nikon Ti2 fluorescence microscope software (NIS-Elements
The area of fluorescent droplet in the daughter micro-well is $182354.1 \pm 4778.2 \, \mu m^2$ (n=576), and the area of fluorescent droplet in the mother micro-well is $91442.7 \pm 4351.3 \, \mu m^2$ (n=64).
Supporting Figures and Table

Figure S1. Measurement of mixing between mother and daughter microwells. A) mixing between mother and daughter microwells by using fluorescein solution. B) mixing between mother and daughter microwells by using a DNA template of 5034 bp. Error bar represents the standard deviation (n=20).
Figure S2. Bright field image demonstrates an eight-step sd-SlipChip that shows the serial dilution pattern of blue food dye.

Figure S3. Analysis of fluorescence intensity from a representative row of an sd-SlipChip device for HBV-plasmid LAMP amplification. A threshold value of 35 RFU was used for all experiments to determine positive vs negative amplification results.
Table S1. Sequence of primers used in the Lamp reaction.

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Nucleotide length</th>
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<tr>
<td>HBV-F3</td>
<td>TCCTCACAATACCGCAGAGT</td>
<td>20</td>
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<tr>
<td>HBV-B3</td>
<td>GCAGCAGGATGAAGAGGAAT</td>
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<tr>
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<td>GTTGGGAAGTGGCAGATTTTGCCTTTTGCTGCTTTTCT</td>
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<td>HBV-FIP</td>
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<tr>
<td>HBV-LF</td>
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<tr>
<td>HBV-BF</td>
<td>AATTTGTCCTGTTATCGCTGG</td>
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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. M. Y., H. Q., L.X., and W. L. performed the experiments; M. Y. and H. Q. performed the data analysis. L. M., R. F. I., and F. S. contributed to generating the concept of serial dilution SlipChip and its application to digital quantification. M. Y., X. C., W. Y. and M. L. contributed to the HBV clinical study. M. Y. and F. S. wrote the manuscript.
References


