Electronic Supplementary Material (ESI) for Analytical Methods.

Supplementary Information

Convenient microfluidic cartridge for single-molecule droplet PCR using common laboratory equipment

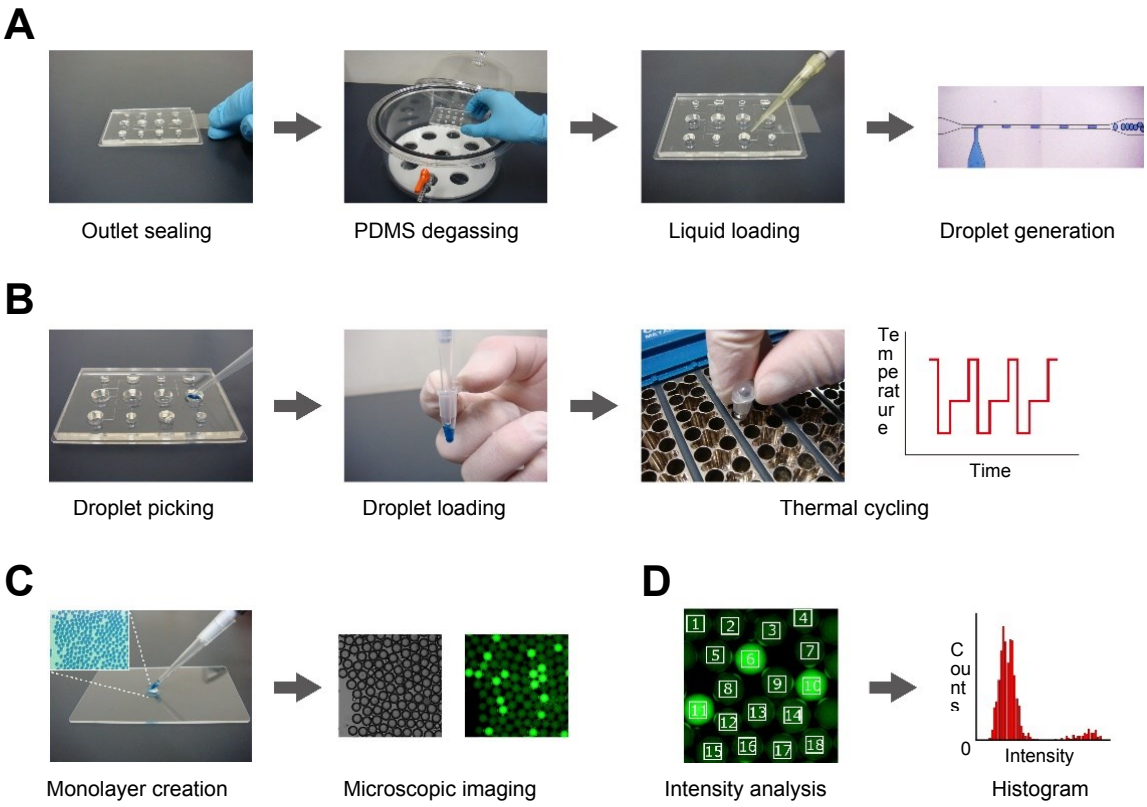
Hirokazu Takahara,[a] Hiroo Matsushita,[a] Erika Inui,[a] Masashi Ochiai[a] and Masahiko Hashimoto*[a]

[a]Department of Chemical Engineering and Materials Science, Faculty of Science and Engineering, Doshisha University, 1-3 Tataramiyakodani, Kyotanabe, Kyoto 610-0321, Japan

***Correspondence:** Prof. Masahiko Hashimoto, Department of Chemical Engineering and Materials Science, Faculty of Science and Engineering, Doshisha University, 1-3 Tataramiyakodani, Kyotanabe, Kyoto 610-0321, Japan
E-mail: mahashim@mail.doshisha.ac.jp
Fax: +81-774-65-6594

Table of Contents

1. Figure S1 Schematic representation of the previous droplet PCR workflow



The sequential experimental procedures in the previous droplet PCR study[1] are schematically shown. (A) Automatic droplet creation, (B) PCR, (C) droplet imaging, and (D) data analysis. For clarity of display, a blue food dye solution was used as the aqueous phase. Adapted with permission from *Electrophoresis*, 2017, **38**, 296–304 (https://doi.org/10.1002/elps.201600309).[1]

2. Materials and methods

2.1. Setup of microfluidic droplet PCR cartridge

Custom PDMS microfluidic sheets (70 mm × 50 mm × 4 mm), fabricated by standard soft lithography, were obtained from YODAKA (Kanagawa, Japan). The structure of the sheets consisted of 50-μm deep channels with a T-junction (see top right of Fig. 1A in the main manuscript), fabricated from PDMS. The T-junction geometry consisted of a continuous phase (oil) channel and a dispersed phase (aqueous solution) channel connected perpendicularly. The channel widths in the current microfluidic chip increased from 50 μm at the T-junction to 250 μm at the up- and down-stream points on the flow path. The channels terminated at their respective reservoirs (refer to R1, R2, and R3 in Fig. 1A of the main text). The capacities of R1, R2, and R3 were 113, 50, and 201 μL, respectively. The diameters of R1, R2, and R3 were 6, 4, and 8 mm, respectively.

A PC plate (70 mm × 50 mm × 4 mm) with a 10 mm diameter through hole, which served as a PCR chamber (R4 in Fig. 1A of the main text), and a thin glass slide (70 mm × 50 mm × 0.15 mm) were bonded together using a thin layer (0.1 mm thick) of double-sided adhesive PET film that possessed a 10 mm diameter punched hole just below R4. The above-mentioned PDMS microfluidic sheet was reversibly adhered to the PC plate through the viscoelastic properties of PDMS. With this arrangement, R3 was located immediately above R4, as shown in Fig. 1C of the main text.

2.2. PCR Reagents

Lambda DNA (New England Biolabs Japan, Tokyo, Japan) was used as template DNA for PCR amplification by adding a defined copy number to 25 μL of solution containing 1× Colorless GoTaq Flexi Buffer (Promega K.K., Tokyo, Japan), 0.5 g/L bovine serum albumin (Sigma-Aldrich Japan, Tokyo, Japan), 5 mM Mg^{2+} (FUJIFILM Wako Pure Chemical Co., Tokyo, Japan), 300 μM dNTPs, 0.2 μM forward and reverse primers (Integrated DNA Technologies (IDT), Coralville, IA, USA), which amplify 76 bp product, 0.2 μM probe DNA (IDT), and 0.625 U GoTaq DNA polymerase (Promega K.K.). The sequences of the primer set and the probe labeled with HEX and BHQ-2 at the 5′ and 3′ ends, respectively, were the same as those used in our previous study.[2]

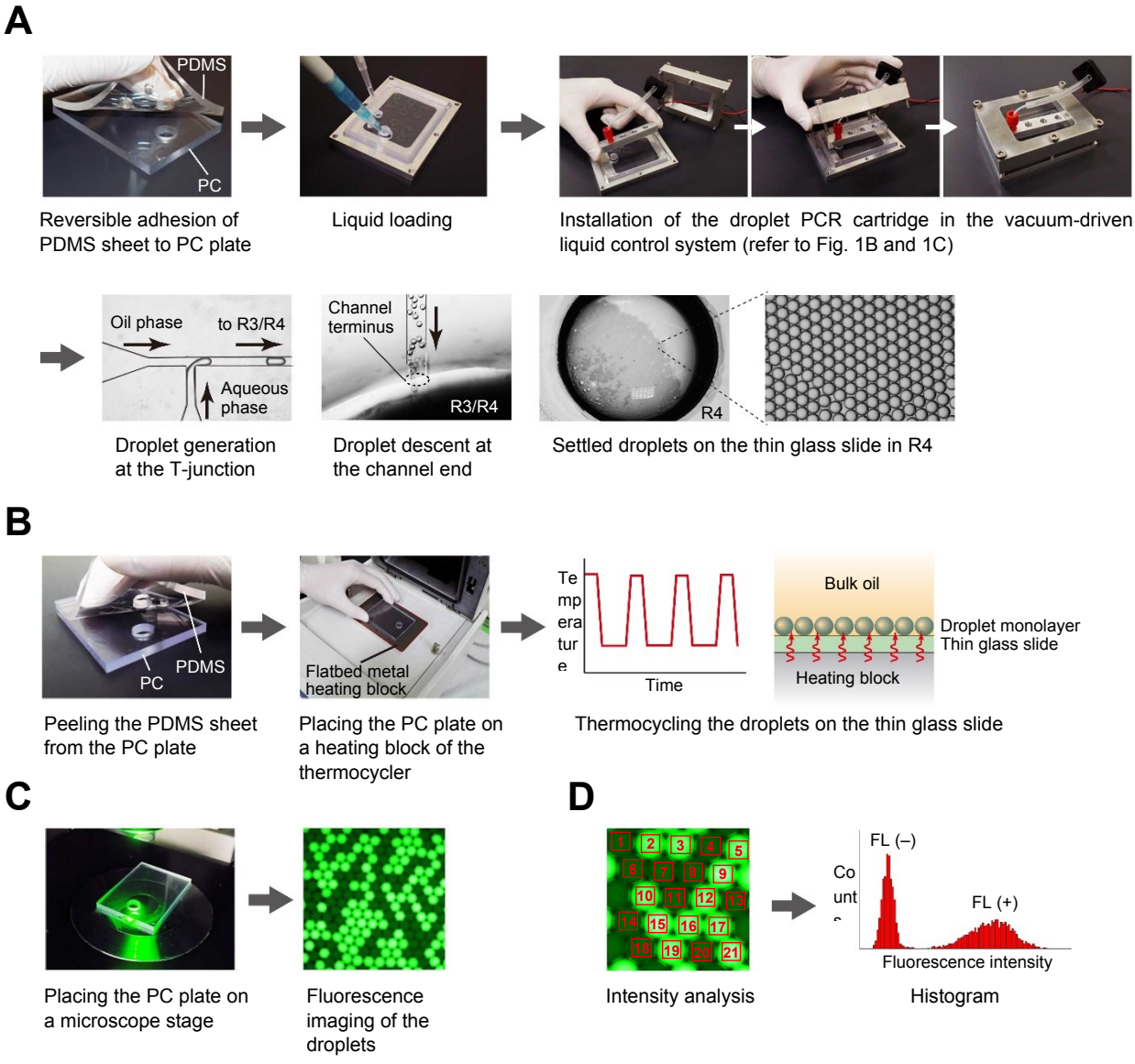
2.3. Operational procedures

Operational procedures involved in the current droplet PCR study is schematically shown in Fig. S2 (see below). First, the above glass/PET/PC/PDMS assembly was put in a vacuum desiccator and degassed at approximately 300 Pa for 15 min to facilitate the loading of fluids into the microchannels.[1] After the device was returned to an atmospheric pressure environment, 40 μL of an oil phase (mineral oil containing 2% (v/v) ABIL EM 90 and 0.05% (v/v) Triton X-100) was dispensed into the inlet reservoir R1 and 25 μL of the PCR mixture

was added to inlet reservoir R2. In addition, 320 μL of the aforementioned oil phase was loaded into R4. Then, the microfluidic device was set in the PZT diaphragm micropump-based fluid manipulation system (see Fig. 1B in the main manuscript) to achieve fluid flows in the microchannels. See our previous publications for full details of the fluid control system.[2, 3] A drive voltage of 250 V_{p-p} at a frequency of 60 Hz was applied to the micropump to remove air from outlet reservoir R3. The pressure difference created between the outlet and inlet reservoirs drew the preloaded oil (in R1, but not in R4) and aqueous solution into the channels, generating W/O droplets at the downstream T-channel junction (see Fig. 2D in the main manuscript).

Approximately 15 min after the onset of droplet generation, the droplet creation was discontinued and the top PDMS layer was peeled off the surface of the PC layer. Before the removal of the PDMS sheet, 160 μL of the oil phase was taken away from R4 to avoid a spill-over of the oil phase from R4. The resultant glass/PET/PC assembly containing the reaction chamber was transferred onto a flatbed heating block of a thermal cycler (Mastercycler nexus flat, Eppendorf Japan, Tokyo, Japan) for 30 cycles of PCR amplification. Fluorescence images of the thermally-cycled droplets were acquired using an inverted fluorescence microscope equipped with an electron multiplier charge-coupled device camera. Fluorescence intensities of droplets present in the imaged field were measured using software bundled with the camera.

2.4. Figure S2 Schematic representation of the current droplet PCR workflow



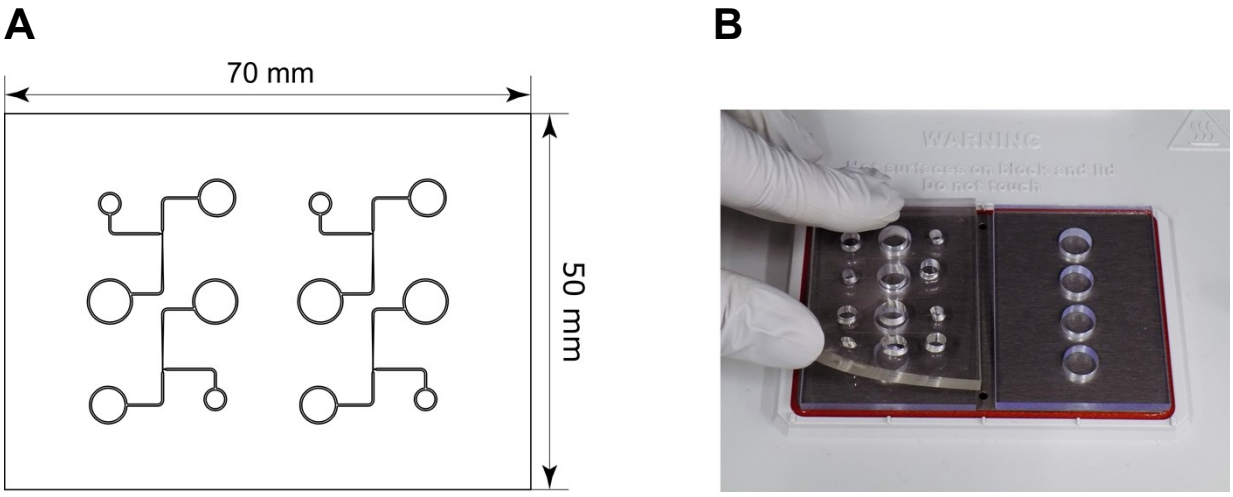
The sequential experimental procedures in the current droplet PCR study are schematically shown. (A) Automatic droplet creation, (B) PCR, (C) droplet imaging, and (D) data analysis.

2.5. Reagents and operational procedures for RT-PCR

Synthetic control RNA that mimics the sequence of SARS-CoV-2 in part (NIHON GENE RESEARCH LABORATORIES K.K., Miyagi, Japan) was used as the template RNA for RT-PCR by adding a defined copy number to 25 μL of solution containing 1× One Step PrimeScript III RT-qPCR mix (Takara Bio K.K., Shiga, Japan), 0.5 μM forward primer (IDT), 0.7 μM reverse primer (IDT), and 0.2 μM probe DNA (IDT). The sequences of the primer set and the probe are identical to those described in a previous paper[4] reported by the National Institute of Infectious Diseases (NIID) in Japan and are as follows: forward primer (NIID_2019-nCOV_N_F2), 5′-AAATTTTGGGGACCAGGAAC-3’; reverse primer (NIID_2019-nCOV_N_R2), 5′-TGGCAGCTGTGTAGGTCAAC-3’; and probe (NIID_2019-nCOV_N_P2), 5′-ATGTCGCGCATTGGCATGGA-3’. The probe was labeled with HEX and Iowa Black FQ (terminal quencher) at the 5′ and 3’ ends, respectively. In addition, the base located 9 bases apart from the 5’-end base in the probe sequence is labeled with an internal ZEN quencher.

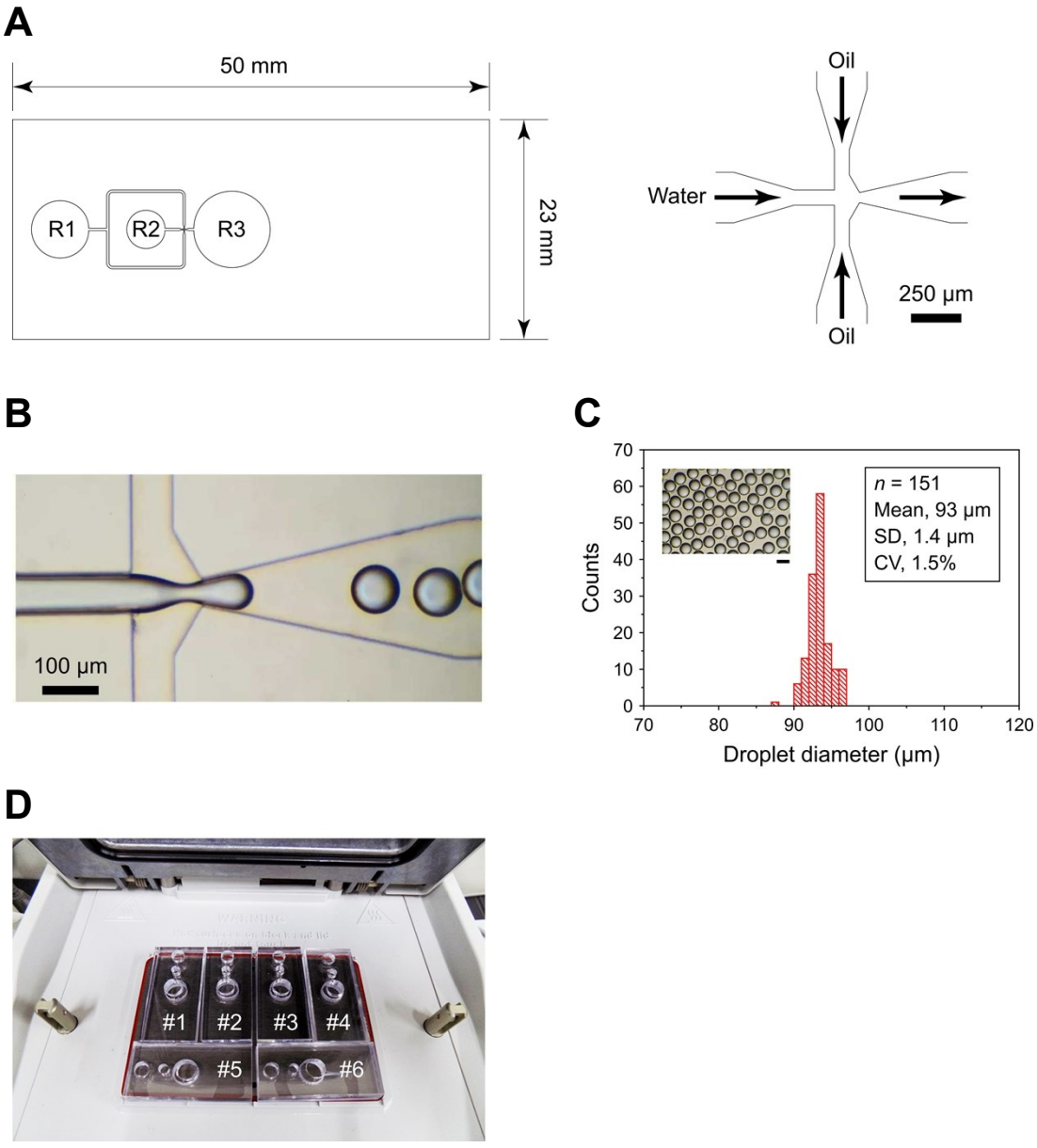
Operational procedures for the droplet RT-PCR are essentially the same as those applied to the lambda DNA testing except for the thermal management applied to the droplets. Details of the thermal management are as follows. After accumulation of droplets in the reaction chamber, for reverse transcription, the glass/PET/PC assembly containing the reaction chamber was placed in a thermostatic oven for 10 min with the temperature maintained at 62°C. Then, the assembly was transferred onto the flatbed heating block of the thermal cycler for temperature cycling (40 cycles of a two-step thermal profile comprising 30 s at 95°C for denaturation and 60 s at 60°C for combined annealing and extension).

3. Figure S3 Configuration of four identical inlet/outlet systems on a single cartridge



(A) Schematic drawing of the four identical inlet/outlet systems configured on the single cartridge and (B) photograph of the two cartridges on the heating block of the thermocycler.

4. Figure S4 Formation of droplets using flow-focusing channel geometry



(A) Microfluidic channel pattern with terminal reservoirs (R1 for oil loading, R2 for aqueous phase loading, and R3 for droplet collection) and an enlarged view of the flow-focusing channel junction (right). A solution of blue food dye was employed as the aqueous phase. (B) Microscopy image showing droplet formation at the cross-junction. (C) Diameter distribution of the analyzed droplets showing total droplet sample size (*n*), mean diameter, SD, and CV. Inset: typical image of the droplet monolayer (scale bar, 100 μm). (D) Photograph of six cartridges arranged on the heating block of the thermocycler.

5. References

1. Y. Nakashoji, H. Tanaka, K. Tsukagoshi and M. Hashimoto, *Electrophoresis*, 2017, **38**, 296–304.
2. N. Okura, Y. Nakashoji, T. Koshirogane, M. Kondo, Y. Tanaka, K. Inoue and M. Hashimoto, *Electrophoresis*, 2017, **38**, 2666–2672.
3. Y. Oda, H. Oshima, M. Nakatani and M. Hashimoto, *Electrophoresis*, 2019, **40**, 414–418.
4. K. Shirato, N. Nao, H. Katano, I. Takayama, S. Saito, F. Kato, H. Katoh, M. Sakata, Y. Nakatsu, Y. Mori, T. Kageyama, S. Matsuyama and M. Takeda, *Jpn. J. Infect. Dis.*, 2020, **73**, 304–307.