Electronic Supplementary Information for

Active droplet-array (ADA) microfluidics enable multiplexed complex bioassays for point of care testing

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1. Materials and reagents

Clinical samples including urine and blood were provided by Department of Laboratory Medicine, Guangzhou First Peoples' Hospital. The clinical samples used in this study were approved by the Ethics Committee of Guangzhou First Peoples' Hospital in accordance with the Helsinki Declaration.

1.1 RNA assay

Solid-phase RNA extraction and real-time RNA isothermal amplification assay kits for *Neisseria gonorrhoeae* (NG), *Chlamydia trachomatis* (CT), *Mycoplasma genitalium* (MG) and *Ureaplasma urealyticum* (UU) were obtained from Shanghai Rendu Biotechnology Co., Ltd., China. The assay kits are comprised of oligo-dT modified magnetic beads suspension, urine storage buffer containing lytic reagents, washing buffer, reaction buffer, detection solution containing primers and fluorescent probes. An enzyme solution with M-MLV reverse transcriptase and T7 RNA polymerase were also included. All positive controls included in the assay kits claim a corresponding RNA concentration of 10^4 copies per μL. With minor modifications, an ethanol-free washing buffer was prepared with 1×PBS buffer and 0.02% Tween-20 (v/v) was used on the chip instead of the washing buffer that included in the assay kits. This allow compatibility with droplet manipulation. Mineral oil, Tween-20 and phosphate buffered saline (PBS) were purchased from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China).

For reagent preloading, mineral oil was used to fill the microwells and the connecting micro-slits. This prevented contact between the reagents and the surface. Unless otherwise indicated, 20 μL of oligo-dT modified MBs suspension, and 50 μL of urine storage buffer containing lytic reagent were loaded into the #1 wells. Then, 50 μL of ethanol-free washing buffer are added to the #2 and #3 wells, and 12 μL of reaction mixture containing buffer, dNTPs, NTPs, primers and fluorescent probes were added into the #4 wells. Three μL of enzyme solution with M-MLV reverse transcriptase and T7 RNA polymerase was added into the #5 wells. Once the aqueous reagents were added, the hydrophobic surface and the mineral

oil pre-loaded into each well ensure that they were maintained in a water-in-oil droplet format due to the surface tension.

1.2 Chemiluminescence immunoassay

The mouse monoclonal antibodies (cat. Nos. M010101, M010102, M010202, and M010203) specific to C-reactive protein (CRP) and procalcitonin (PCT), as well as the corresponding standard antigens (N010101 and R010201) were brought from Hangzhou Biogenome Biotech Co, Ltd.. One set of the antibodies was conjugated with the magnetic beads as the capture beads (MB-Ab), and the others were conjugated with the horseradish peroxidase (HRP) to detect the corresponding target proteins (HRP-Ab) and generate chemiluminescence (CL) signal by an enzymatic reaction with the HRP substrate. The magnetic beads (BeaverBeads™ MG COOH, 2.0 μm) were purchased from Beaver Nano-technologies Co., Ltd., and HRP was obtained from Sangon Biotech Co., Ltd.

The dilution buffer contains 0.5% bovine serum albumin (BSA, Invitrogen), 0.25% Tween20, 250 ppm Proclin300 (Baiyanbio, Shanghai, China), and 0.01 mol·L^{-1} PBS (pH7.4). The HRP substrate including luminol and hydrogen peroxide (H_2O_2) was purchased from Keyuebio (Beijing, China). The washing buffer with 0.25% Tween20 in 0.01 mol·L^{-1} PBS (pH 7.4) was obtained from Hexin Biotechnology (Guangzhou, China). To determine the dynamic range of the CL immunoassay for CRP and PCT, the corresponding antigen was diluted two times (10%, v/v) with dilution buffer. It was then spiked in normal human serum to a final concentration of 0.2-2000 ng·mL^{-1} or 0.05-60 ng·mL^{-1}.

To prepare a CL immunoassay chip with preloaded reagents, mineral oil was first added to fill the microwells, followed by serials of aqueous reagents injected into the corresponding wells. Here, the 27 μL of reaction mixture for CRP includes 4 μL of MB-Abs (20 μg·mL^{-1}), 3 μL of HRP-Abs (1.7 μg·mL^{-1}), and 20 μL of dilution buffer. The mixture for PCT contains 5 μL of MB-Abs (25 μg·mL^{-1}), 5 μL of HRP-Abs (1.7 μg·mL^{-1}), and 20 μL of dilution buffer. This was added into each #1 well. Each washing well (#1 or #2) had 60-μL washing buffer. The last two wells had 20-μL HRP substrate with equal volumes of luminol and H_2O_2. These aqueous reagents formed water-in-oil droplets and could be confined individual wells.

2. Droplet Chip

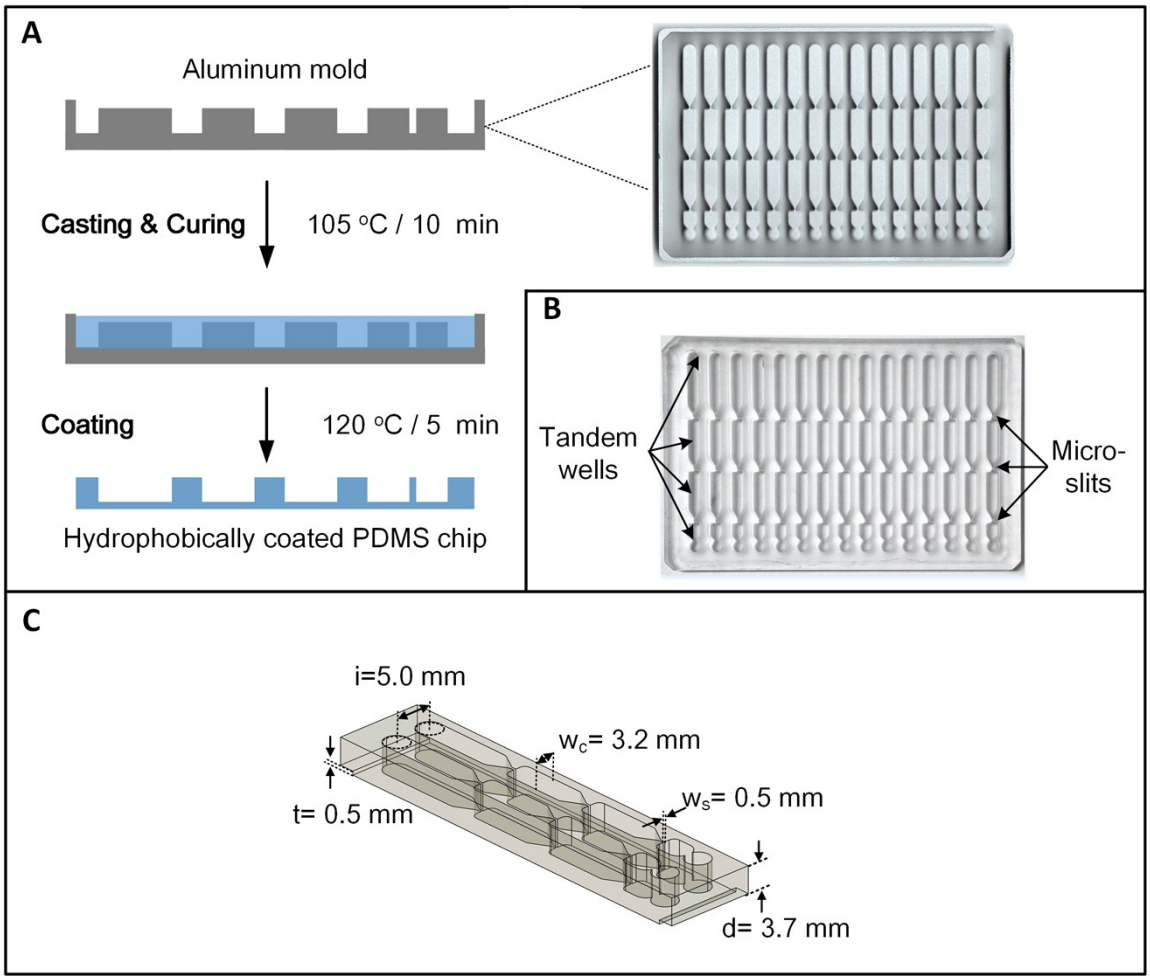

Fig. S1 (A) The fabrication diagram for the polydimethylsilane (PDMS) droplet chip. The microchip was designed using AutoCAD and translated into a negative aluminum mold (inset) machined by computer numerical control. The PDMS precursor was prepared at a 1:10 crosslinker-to-base ratio (Sylgard 180, Dow Corning Corp.). This was subsequently poured into this mold and incubated at 105 °C for 10 min. Finally, the surface of the microwells and the micro-slits were treated with 0.3 % (w/v) of Teflon AF1600 (DuPont Fluoroproducts, Wilmington, DE) dissolved in a FC-40 solvent (Fluorochem, Derbyshire, UK) to form a stable hydrophobic coating. (**B**) Photograph of the droplet chip. It has a footprint of 8.5 × 5.4 cm^2, which is approximately the size of a credit card. (**C**) Sketched layout of the droplet chip with detailed parameters. Here, *i* is the total spacing from well canter to canter, *Wc* is the width of the wells, *Ws* is the width of the micro-slits, *d* is the width of the wells, and *t* is the typical thickness of the bottom.

3. Large-volume droplet anchorage

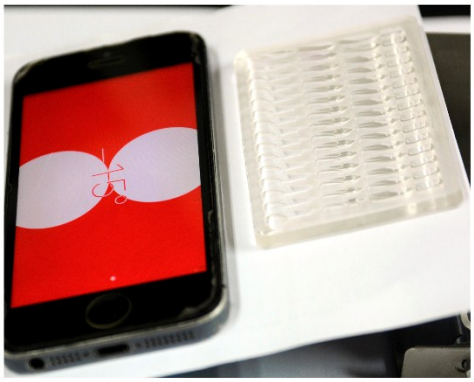

Fig.S2 Robustness of large-volume droplet anchorage under incline. Here, 0.5 % phenolphthalein solution and 1 mol·L^{-1} NaOH with 0.01~0.025 % Tween 20 (v/v) to mimic liquid reagents were individually added into the neighboring wells for visualization of droplet crosstalk. For incline tests, the chip was placed on an adjustable slope with a slope angle indicated by a Level-Meter app (iPhone 5). We investigated the movement or leakage of oil and droplet from the wells by carefully changing the incline angle. For vibratory tests, the chip was placed on an orbital shaker. The chip was shaken by an increasing rotational frequency, with the naked eye to confirm the color change of the droplet. The result showed that the tandem wells experienced 600 revolutions per minute (RPM) without reagent cross-talk (†**Video S1**).

4. Reproducibility of microfluidic CL immunoassay on the droplet chip

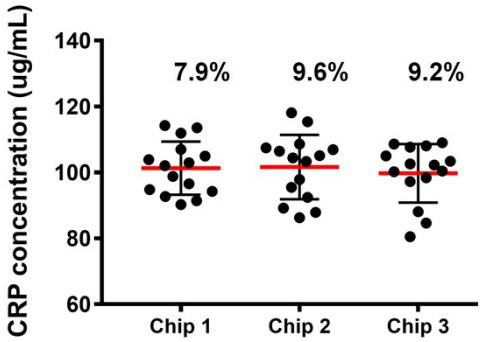

Fig.S3 Reproducibility for quantitative detection of CRP on the droplet chip.