Supporting Information for

Digital Isothermal Quantification of Nucleic Acids via Simultaneous Chemical Initiation of Recombinase Polymerase Amplification (RPA) Reactions on SlipChip

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Table of Contents:

Experimental section:

Chemicals and Materials

All salts and solvents purchased from commercial sources were used as received unless otherwise stated. The TwistAmp^{TM} exo kit for RPA was purchased from TwistDx Limited (Cambridge, United Kingdom). The RPA primers and probe for detection of MRSA *mecA* gene were generously provided by TwistDx Limited. Bovine serum albumin (BSA) solution was ordered from Roche Diagnostics (Indianapolis, IN). Tetradecane, chloroform, acetone, ethanol, and DEPC-treated and nuclease free water were obtained from Fisher Scientific (Hanover Park, IL). Dichlorodimethylsilane was purchased from Sigma-Aldrich (St. Louis, MO). Soda–lime glass plates coated with photoresist and chromium were ordered from Telic Company (Valencia, CA). Spectrum food colors (brown and blue food dye) were obtained from August Thomsen Corp (Glen Cove, NY). Photomasks were ordered from CAD/Art Services, Inc. (Bandon, OR). PCR tubes and barrier pipette tips were purchased from Molecular BioProducts (San Diego, CA). All PCR primers were purchased from Integrated DNA Technologies (Coralville, IA). SsoFast EvaGreen Supermix (2X) was obtained from Bio-Rad Laboratories (Hercules, CA). Methicillin-resistant *Staphylococcus aureus* [MRSA], ATCC 43300, was purchased from American Type Culture Collection (Manassas, VA) and MRSA gDNA was purified according to the manufacturer's recommendations using Qiagene Puregene Yeast/Bact. Kit A obtained from Qiagen (Valencia, CA).

Fabrication of SlipChip for digital RPA

The procedure for fabrication of SlipChip from soda lime glass was based on the methods developed previously.[1] In order to fabricate SlipChip with wells of different depths, the following procedures were used: 1) The glass plate coated with chromium and photoresist was aligned with a photomask containing the design for Type I wells, Type II wells, and ducts by using a mask aligner, then the photoresist layer was exposed to UV light using standard exposure protocols. 2) After exposure, the glass plate was immersed in 0.1 mol/L NaOH solution to immediately remove the photoresist from exposed areas. 3) The exposed underlying chromium layer was removed by using a chromium etchant (a solution of 0.6:0.365 mol/L $HClO_4$ / $(NH_4)_2Ce(NO_3)_6$). 4) The glass plate was then thoroughly rinsed with Millipore water and dried with nitrogen gas. 5) The 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 photoresist layer and chromium coating were removed in the previous steps. 6) The glass plate was thoroughly washed with Millipore water and dried with nitrogen gas. 7) The glass plate was aligned with a second photomask containing the design of satellite wells and was exposed to UV light by using the standard exposure protocols. Then steps 2) to 6) were repeated. Finally, the remaining photoresist was removed by using acetone, and the underlying chromium layer was removed by using the chromium etchant. The etched depth was controlled by the etching time and speed, which was controlled by the etching temperature. The Type I and Type II wells were etched to be 50 μm deep, and the satellite wells were etched to be 15 μm deep. The

volume of Type I wells, Type II wells, and satellite wells were 6 nL, 3 nL, and 0.2 nL respectively.

The glass plate was oxidized in a plasma cleaner (Structure Probe, Inc., West Chester, PA) for 10 minutes and then immediately transferred into a desiccator (Fisher Scientific, Hanover Park, IL). Dichlorodimethylsilane (50 μL) was injected into the desiccator and then a vacuum was applied to perform gas-phase silanization for one hour. The silanized glass plate was thoroughly cleaned with chloroform, acetone, and ethanol, and then dried with nitrogen gas. The silanized glass plate was used for digital RPA experiments within one day. The glass plate was reused after thoroughly cleaning with piranha solution (3:1 sulfuric acid : hydrogen peroxide) and silanized with the procedure described above.

Assembling the SlipChip

The SlipChip was assembled under tetradecane. The tetradecane was de-gassed before digital RPA experiments. The bottom plate was first immersed into tetradecane in a Petri dish, with the patterned wells facing up. The top plate was then immersed into tetradecane 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 1 and stabilized using binder clips.

Digital RPA on the two-step SlipChip with on-Chip initiation

The RPA master mixture was prepared by rehydrating the lyophilized enzyme mixture in 29.5 μL of rehydration buffer and 10 μL of water, then adding 3.5 μL each

of RPA primers A and B (10 μM each) and 1 μL of the *mecA*-specific probe (TwistDx Ltd). The solution was pulse-vortexed three times and sonicated in a FS60H (Fisher Scientific) at room temperature for 10 minutes. 5 μL of BSA solution (20 mg/ml) was added to the RPA master mixture. For experiments with on-chip initiation of digital RPA (Figures 3, 4, 5, 6, 7), 1.5 μL of MRSA gDNA template solution ($1:10^5$ to 1:10 dilution) was added to 28.5 μL of the RPA master mixture as Reaction Mixture 1 (Figure 1, orange line). 4 μL of 280mM of $Mg(OAc)_2$ solution was added to 15 μL of the RPA master mixture as Reaction Mixture 2 (Figure 1, blue line). Reaction Mixture 1 was loaded into the SlipChip by pipetting (Figure 2C-D), and then the top plate was slipped relative to the bottom plate to compartmentalize the gDNA template in Reaction Mixture 1. Then Reaction Mixture 2 was injected into the SlipChip (Figure 2E), and the top plate was slipped again in the same direction relative to the bottom plate to overlay the Type I and Type II wells and to initiate the digital RPA reaction simultaneously (Figure 2F). Figure S1 shows the step-by-step loading procedure using food dyes. The SlipChip was placed on a metal adaptor and incubated for 1 hour at 39℃ (experiments in Figure 3, 4, 5, 6, 8B, 8D).

RPA on the one-step SlipChip with on-Chip initiation

The RPA master mixture was prepared by rehydrating the lyophilized enzyme mixture in 29.5 μL of rehydration buffer and 10 μL of water, then adding 3.5 μL each of RPA primers A and B (10 μM each) and 1 μL of the *mecA*-specific probe (TwistDx Ltd). The solution was pulse-vortexed three times and sonicated in a FS60H (Fisher Scientific) at room temperature for 10 minutes. 5 μL of BSA solution (20 mg/ml) was

added to the RPA master mixture. 1.5 μL of MRSA gDNA template solution ($1:10^4$ dilution) was added to 28.5 μL of the RPA master mixture as Reaction Mixture 1 (Figure 1, blue line). 4 μL of 280mM of $Mg(OAc)_2$ solution was added to 15 μL of the RPA master mixture as Reaction Mixture 2 (Figure 1, orange line). Reaction Mixture 1 and Reaction Mixture 2 were introduced simultaneously into the one-step SlipChip by applying pressure as described before.[2] One slipping step broke both fluidic paths and compartmentalized the loaded reagent. At the same time, the Type I wells were overlaid with Type II wells to initiate the reaction (Figure 6).

RPA on the two-step SlipChip with pre-initiation

For experiments with pre-initiation (Figures 1, 7A, and S2), 5μL of BSA solution (20 mg/mL) and 4 μL of $Mg(OAc)_2$ solution (280mM) were added to 48 μL of the RPA master mixture. A solution of 1.5 μL of MRSA gDNA template solution ($1:10^4$ dilution) was added to 28.5 μL of this reagent mixture as Reaction Mixture 1. The remaining solution was treated as Reaction Mixture 2. Reaction Mixture 1 was incubated at room temperature (approximately 25℃) for 1 min, and then injected into the SlipChip by pipetting (Figure 2C-D). The entire loading procedure took 4 minutes under room temperature. The top plate was slipped relative to the bottom plate to compartmentalize the RPA solution (Figure 2E). Reaction Mixture 2 was then loaded into the SlipChip by pipetting (Figure 2E). The top plate was slipped again relative to the bottom plate and the Type I wells were overlaid with Type II wells (Figure 2F) The SlipChip was then placed on an adaptor for incubation at 39 ℃ for one hour.

Quantitative RPA by using a plate reader

The RPA master mixture was prepared as described above. 5μL of BSA solution (20 mg/mL) and 4 μL of $Mg(OAc)_2$ solution (280mM) were added to 48 μL of the RPA master mixture. Then 19 μL of the reaction mixture was placed in a well of a 96 well plate, and 1 μL of MRSA gDNA template solution ($1:10^3$ dilution) was added to each well. The well plate was immediately placed in a plate reader (BMG LABTECH, Germany) with temperature controlled at 25℃. For the control experiment without template, after loading the reaction mixture into a 96 well plate, 1 μL of water was added to each well instead of gDNA template solution (Figure 1, orange line). For the control experiment without $Mg(OAc)_2$ solution, 5μL of BSA solution (20 mg/mL) and 4 μL of water were added to the RPA master mixture. Then 19 μL of the reaction mixture was placed in a well of a 96 well plate, and 1 μL of MRSA gDNA template solution ($1:10^3$ dilution) was added to each well (Figure 1, blue line). Fluorescence intensity was acquired every minute for two hours. A shaking step of 2 seconds was applied after each acquisition cycle.

Digital PCR on the SlipChip

This digital RPA SlipChip was designed to be compatible to perform digital PCR as well (Figure S3). For digital PCR, the PCR reaction master mixture consisted of 20μL of SsoFast EvaGreen SuperMix (2X), 2μL of BSA solution (20 mg/mL), 15μL of water, and 1μL of each forward and reverse primers (10μM each). A solution of 1.5μL of MRSA gDNA template ($1:10^4$ dilution) was added to 28.5μL of the above

reaction mixture. The primers for detection of *mecA* gene in MRSA gDNA were: primer 1, CAA GAT ATG AAG TGG TAA ATG GT; primer 2, TTT ACG ACT TGT TGC ATA CCA TC.

The PCR reaction mixture was injected into the SlipChip to fill all the Type I wells (Figure S3D). Without loading the second reagent, the top plate was slipped relative to the bottom plate to directly overlay the Type I wells with the Type II wells (Figure S3E). The Type II wells, which were previously filled with tetradecane during assembly of the SlipChip, offered additional volume for thermal expansion during PCR thermal cycling. The SlipChip was then placed on an adaptor in the Mastercycler for thermal cycling.

An initial step at 95 ℃ (2 min) was used to activate the enzyme for reaction. Next, a total of 35 cycles of amplification were performed: denaturation step of 1 min at 95 ℃, annealing step of 30 sec at 55 ℃, and a DNA synthesis step of 30 sec at 72℃. After the final cycle, a final DNA extension step was performed for 5 min at 72℃.

Image acquisition and analysis

All fluorescence images were acquired by using a Leica DMI 6000 B epi-fluorescence microscope (Leica Microsystems, Germany) with a 5X / 0.15 NA objective and L5 filter. All fluorescence images were corrected by a background image obtained with a standard fluorescent slide. Fluorescence images were stitched together by using MetaMorph software (Molecular Devices, Sunnyvale, CA).

References:

(1) Du, W. B.; Li, L.; Nichols, K. P.; Ismagilov, R. F. *Lab Chip* **2009**, *9*, 2286-2292.
(2) Li, L. A.; Karymov, M. A.; Nichols, K. P.; Ismagilov, R. F. *Langmuir* **2010**, *26*, 12465-12471.

Figures:

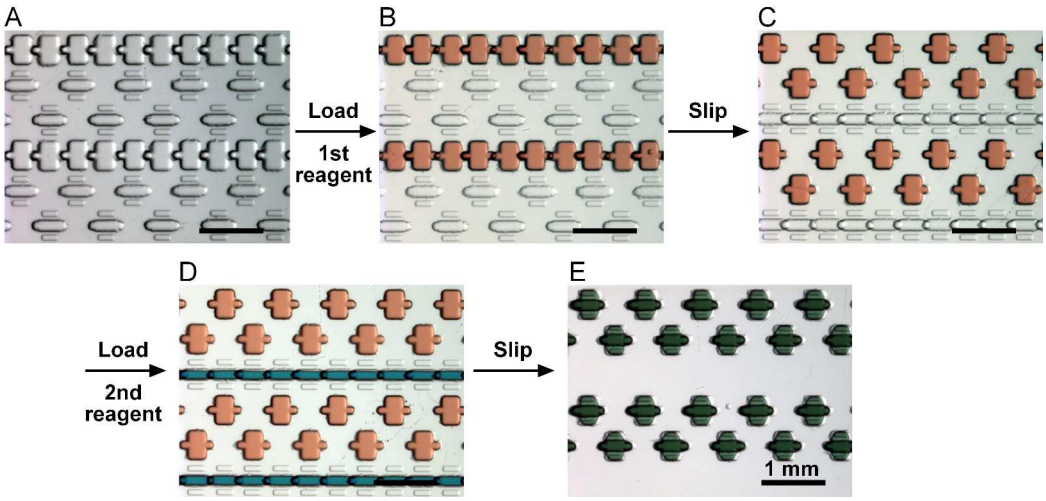

Figure S1. Food dye experiment demonstrated the operation of slipping for digital RPA SlipChip. A) The top and bottom plates of the digital RPA SlipChip were aligned to form the continuous fluidic path by overlapping the Type I wells. B) The first reagent (pink) was loaded into the SlipChip by pipetting. C) The top plate was slipped relative to the bottom plate to compartmentalize the reagent loaded in the Type I wells, and a second fluidic path was formed by overlapping the Type II wells D) The second reagent (blue) was loaded into the SlipChip by pipetting. E) The top plate was slipped again relative to the bottom plate, and the Type I and Type II wells were overlaid to combine the two reagents.

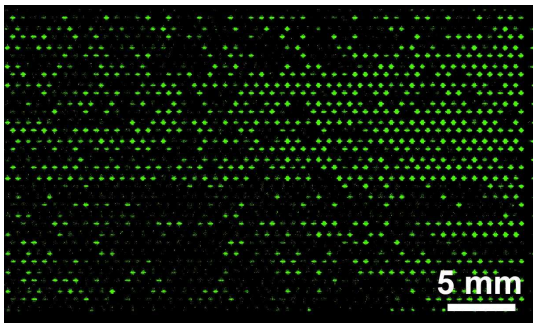

Figure S2. A "streaky" distribution of positive wells was obtained when RPA is pre-initiated off-chip for one minute and loaded onto the chip via pipetting over 4 minutes. This result indicates that the amplification reaction is proceeding as the reaction mixture is being loaded. MRSA gDNA (at concentration of $1{:}10^4$ dilution) was pre-mixed with Reaction Mixture 1 (containing magnesium acetate). The pre-initiated mixture was loaded into Type I wells from the right side of the digital RPA SlipChip as described in Figure 2.

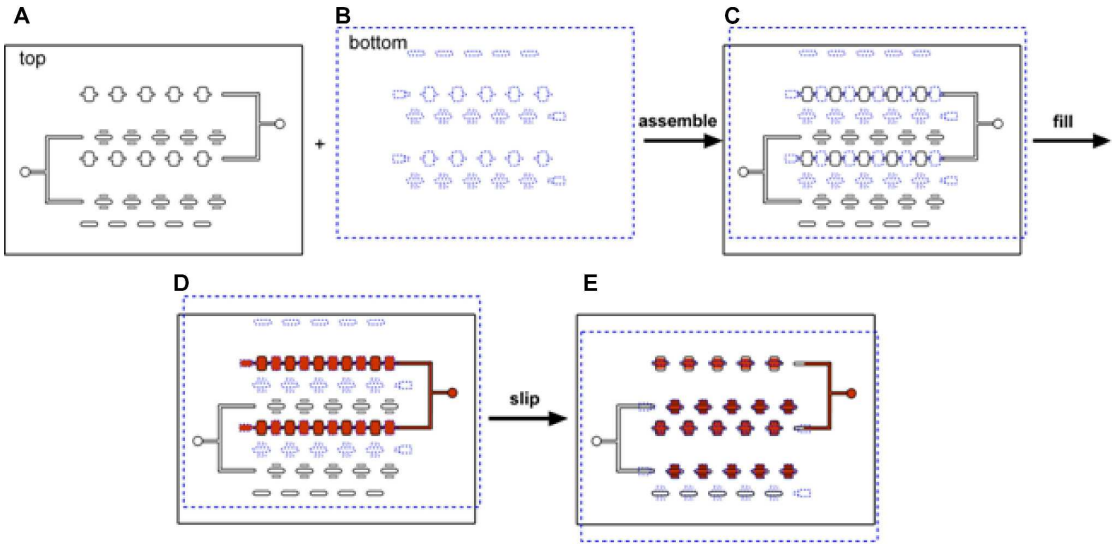

Figure S3. Schematic drawing shows the procedures to perform digital PCR by using the two-step SlipChip. A) top and B) bottom plate of the SlipChip. C) Assembly of top and bottom plates to establish the first continuous fluidic path of Type I wells. D) Loading of the PCR reagent (red). E) One-step slipping to compartmentalize the PCR reagent and overlap with Type II wells.

Author Contributions:

F.S., E.K.D, W.D., J.E.K, and R.F.I designed the SlipChip experiments and wrote the paper. F.S. and E.K.D. performed experiments. J.E.K performed statistical analysis. F.S. and W.D contributed to SlipChip design. E.K.D. optimized experimental conditions. O.P. provided the primers and probe, developed and produced by the TwistDx team, for amplification and detection of *mecA* gene in Methicillin-resistant *Staphylococcus aureus* (MRSA). He also provided advice on RPA protocols and co-wrote the description of the mechanism of amplification and fluorescent detection for recombinase polymerase amplification (RPA) in the manuscript.