Supporting Information

Agarose Droplet Microfluidics for Highly Parallel and Efficient Single Molecule Emulsion PCR

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Materials

Glass (B270 glass, 150 um thick) for microfluidic chip fabrication was purchased from Shaoguang Company Co., Ltd (Changsha, China). 749 FLUID and 5225C FORMULATION AID were purchased from Dow Corning Company. Silicone oil AR 20 (10836-500 ML), octadecyltrichlorosilane (OTS, 104817-25G) and agarose (A2576-5G, ultra-low gelling temperature) were purchased from Sigma-aldrich (St. Louis, MO). Sodium periodate and sodium cyanoborohydride were purchased from Alfa Aesar (Ward Hill, MA). Lambda DNA was supplied by Fermentas (Canada). Hot-start Blend Taq plus polymerase and dNTPs were supplied by TOYOBO (Japan). 5' end modified forward primer 5'-NH2-(C18)2-TAAGCACGAACTCAGCCAGAACGA-3', forward primer 5'-TAAGCACGAACTCAGCCAGAACGA-3' and reverse primer 5'-CAAGCTTTGCCACACCACGGTATT-3' for lambda DNA PCR were synthesized in house. SYBR Green□(20×, 500 uL, Biovision bio-technology Co., Ltd) was applied to stain PCR product in agarose beads.

Experimental Section

Agarose Melting and Geling Curve Measurement The melting and geling curve of agarose were determined by measuring the absorbance of 2% (w/v) agarose solution at 260 nm with a UV-Visible Spectrophotometer (Agilent 8453, USA). 0.04 g agarose was suspended in 2 mL deionized water and stirred during the whole measuring process. The temperature was set from 10°C to 75°C and back to 10°C by 0.1°C step and held for 30 sec each step.

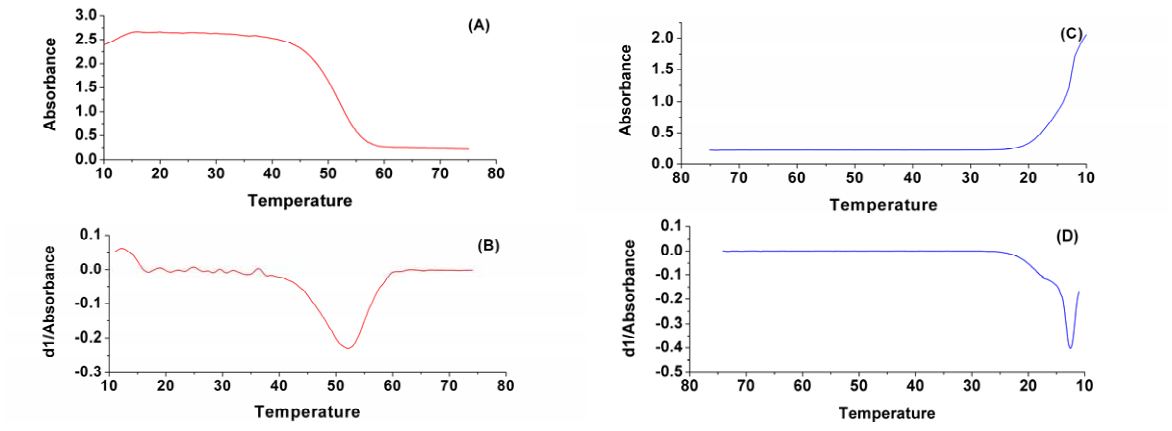

Figure S1. Melting curve of agarose (A) and its relevant first-order derivative curve (B), and gelling curve of agarose (C) and its relevant first-order derivative curve (D).

Microfabrication The crossed-channel patterns were drawn using an AutoCAD software and then printed to a dark mask film. The microchannels on the mask were photolithographically transferred onto glass surface, and etched to produce a 360 μm (w) × 230 μm (d) cross-channel in a well-stirred bath containing dilute HF/HNO_3 solution. Four 1.8-mm-diameter holes were drilled on the etched glass at channel terminals using a mechanical drill (TBM115, Proxxon). The etched glass was thermally bonded at 580°C for 2h to an identical thick featureless glass in a Muffle furnace (Ney Centurion Q50, US). After bonding, the microchannels were rinsed with isopropanol, acetone, piranha solution (H_2SO_4/H_2O_2, 3/1) and deionized water respectively and dried with nitrogen gas. Following these, glass channels were hydrophobically treated with a 0.1% solution of octadecyltrichlorosilane in dry toluene for 5 min. The treated channels were rinsed by dry toluene, isopropanol and deionized water orderly.

Relative PCR Efficiency in Different Concentrations of Agarose Solutions To study whether agarose would inhibit PCR, PCR was performed in different concentrations of agarose solutions. The thermal cycling conditions were as follows: 94°C for 3 min (initial denaturation), 25 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec, followed by a single final extension for 5 min at 72°C in a peltier thermal cycler (BIO-RAD, Richmond, CA). PCR products were analyzed by a 2.5% agarose gel electrophoresis with two replicates to reduce random error. The relative PCR efficiency of each product was determined according to the intensity of electrophoretic band using ImageJ software. PCR efficiency in 0% agarose solution was set to 1.0 and efficiency of the other samples was compared relatively. Agarose of lower concentration has lower mechanical strength after transformed to gel beads. For example, microbeads of 1% agarose concentration were severely broken into pieces after the routine washing procedure. Therefore we choose 2% as the optimized concentration of agarose in droplets to achieve both high PCR efficiency and mechanical strength of agarose beads.

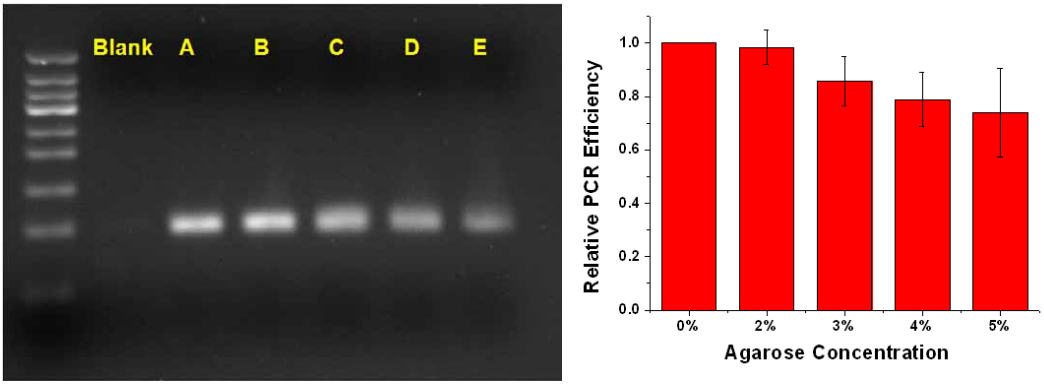

Figure S2. Electrophoresis image of PCR product of samples with different agarose concentration (left) and comparison of relative PCR efficiency in agarose solutions at different concentrations (right).

Primer Conjugation Forward primer was conjugated to agarose matrix using Schiff-base reaction. The 5' end modified forward primer (5'-NH_2-$(C18)_2$-TAAGCACGAACTCAGCCAGAACGA-3') was synthesized using a Polygen 12 DNA synthesizer (Munich, Germany), purified by RP-HPLC (Agilent 1100, USA) and desalted using a NAP column (GE Healthcare, Piscataway, NJ). 125 μL 4% agarose solution prepared at 75°C was mixed with 3.4 mg $NaIO_4$ and cooled to room temperature naturally. After being solidified at 4°C, the activated agarose gel was washed in deionized water three times for 30 min each, then melted and mixed with 125 μL conjugation buffer (0.15 M NaCl, 0.1 M $NaHCO_3$,

pH 8.5) containing 3.14 mg $NaCNBH_3$ and 32 nmole 5' end modified forward primer. After 6 h reaction, the activated agarose was conjugated with primer and cooled to solidify. Free primer was removed by mean of electrophoresis (120 V, 45 min). To confirm the success of the coupling, PCR was carried out using agarose conjugated forward primers and the PCR product was tested in 2.5% agarose gel electrophoresis. If the primer is successfully tethered to agarose polymer chains, PCR will produce amplicons physically attached to agarose polymer chains which will not migrate in gel eletrophoresis. As shown in Figure S3, the amplification product barely moved under electrophoresis, suggesting successful coupling of forward primer to the polymer. Such a physical attachment of amplicons to agarose prevents their diffusion out of agarose, permitting long term process of agarose beads without worrying about the leakage of DNA product.

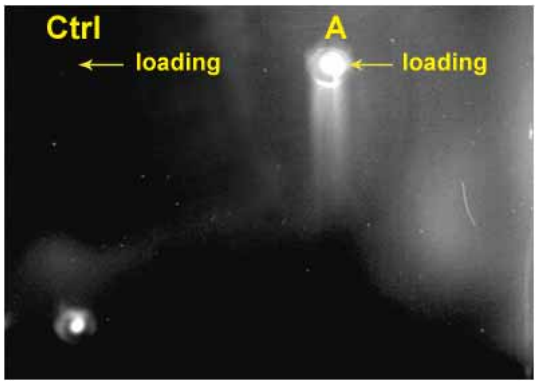

Figure S3. The electrophoresis image of PCR product amplificated with free forward primer (left channel) and agarose conjugated forward primer (right channel). The arrows indicate loading spots of the two samples.

ePCR Procedure The droplet PCR cocktail was prepared by thoroughly mixing 2% agarose, 0.1 U/μL Hot-start Blend Taq plus DNA polymerase, Blend Taq buffer, 0.2 mM dNTPs each, 0.4 μM primers (5'-agarose-$(C18)_2$-TAAGCACGAACTCAGCCAGAACGA-3', 5'-CAAGCTTTGCCACACCACGGTATT-3') amplifying a 101 bp sequence of lambda DNA fragment and specified amounts of template. In the condition of 0.4/5.0 mL/hr (aqueous/oil) velocity, the generated droplets had diameter of 80±5 μm and were collected in a tube until 100 μL of the aqueous phase was collected. The thermal cycling conditions were as follows: 94°C for 3 min (initial denaturation), 25 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec, followed by a single final extension for 5 min at 72°C in a peltier thermal cycler. The amplified emulsions were solidified to agarose beads by cooling to 4°C. After removing the oil phase by acetone, isopropanol and deionized water sequentially, the agarose beads were stained by dispersing in 100 μL deionized water with 6 μL 20× SYBR Green.

Single copy PCR fluorescence imaging was performed with inverted fluorescence microscope (Nikon Eclipse Ti-U, Japan). About 300 beads from each sample were exposed for 2 sec at 100 fold magnification and the maximum intensity of each bead was measured using NIS-Elements BR imaging software. Background intensity was determined according to the intensity of the brightest microbead from the control experiment. Beads with fluorescence intensity higher than the background intensity were counted as the positive beads produced from a droplet carrying at least one DNA template.

Quantification of Droplet PCR Efficiency A real-time quantitative PCR assay was performed in order to acquire the quantity of DNA in each agarose bead after 25 cycles of PCR amplification from single DNA template. The reaction

mixture was prepared according to the following final concentrations: 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM of each primer, 250 mM of dNTPs, 0.125 unit of Taq polymerase and 1× SYBR Green. The copies of template DNA in four standard samples were 6.0E+06, 6.0E+07, 6.0E+08, 6.0E+09 separately. For four unknown samples, 1 mL of agarose beads solution (include 100 beads) was added. To reduce random errors, each sample has 3 replicates in the experiment on a StepOne™ Real-Time PCR System from Applied Biosystems. Q-PCR resutls indicated that the average numbers of amplicons in each microbead after amplified from template concentration of 0.15, 0.5, 1.5 copy/bead were about 2.0 E+7, 4.68 E+6, 4.03E+6 respectively. Correspondingly, the PCR efficiency ($E= (N^{1/25}-1) *100\%$) in agarose droplets with template concentration of 0.15, 0.5, 1.5 copy/bead, as calculated was 96%±5%, 85%±7% and 84%±7% respectively.

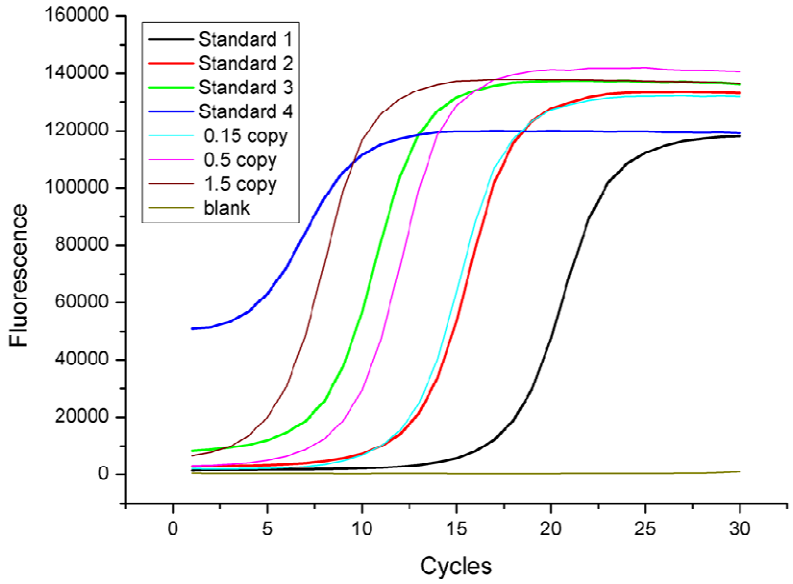

Figure S4. Q-PCR analysis results of amplicons in agarose beads amplified from 0.15, 0.5, and 1.5 copy of template/bead.