Supporting Information

Massively Parallel Single-Molecule and Single-Cell Emulsion Reverse Transcription Polymerase Chain Reaction Using Agarose Droplet Microfluidics†

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Synthesis of RNA/DNA sequences. RNA template, forward primer and reverse primer for RT-PCR were synthesized in house and their sequences are listed in Table 1S. The DNA product was synthesized on a 12-Column DNA Synthesizer (PolyGen GmbH). Except for normal DNA bases, 5′-acrylic groups were also synthesized on the machine. The synthesis protocol was set up according to the requirements specified by the reagents' manufacturers. For 5′-acrylic group coupling, a coupling time of 600s was used to ensure coupling efficiency. Following on from machine synthesis, the DNA product was deprotected and cleaved from CPG by incubating with 0.4 mL ammonium hydroxide for 8 hours at 65°C in a water bath. The cleaved product was transferred into a 2mL centrifuge tube and mixed with 40 μL 3.0 M NaCl and 1.2 mL ethanol, after which the sample was placed into a freezer at -20°C for ethanol precipitation. Afterwards, the DNA product was spun at 14000 rpm at 4°C for 10 minutes. The supernatant was removed, and the precipitated DNA product was dissolved in 500 μL 0.1 M trithylamine acetate (TEAA) for HPLC purification. The HPLC purification was performed with a cleaned Waters C18 column on an Agilent 1100 HPLC machine. The collected DNA product was dried and processed by detritylation, dissolved and incubated in 200 μL 80% acetic acid for 20 minutes. The detritylated DNA product was mixed with 400 μL ethanol and dried with a vacuum dryer. The purified DNA product was quantified by determining the UV absorption at 260 nm, after which the DNA product was dissolved in DI water and stored in the freezer at -20°C for future experiments.

The RNA template was synthesized on ABI 3400 DNA Synthesizer (Applied Biosystems) using the 2′-O-Triisopropylsilyloxymethyl (TOM)-protected RNA monomer phosphoramidites (Glen Research Corp., Sterling, VA). The synthesis cycle is similar to DNA cycle with the specific requirements according to the reagents' manufacturers. The coupling time was set to 6 min to ensure coupling efficiency. Following on from machine synthesis, the RNA product was deprotected and cleaved from CPG by incubating with 1.5 mL AMA (ammonium hydroxide/ 40% aqueous methylamine 1:1) solution

for 10 min at 65°C in a water bath. The use of sterile conditions from this point forward is essential. The cleaved product was transferred into a 2mL centrifuge tube and dried using a stream of Argon. Afterwards, the RNA was fully dissolved in 115μL DMSO and heated at 65°C for 5 min in order to get the RNA into solution. 60μL of TEA (triethylamine) was added and mixed gently. Then, 75μL of TEA.3HF(triethylamine trihydroflouride) was added and the mixture was heated at 65°C for 2.5hr. Immediately before beginning cartridge purification, the deprotection solution was cooled and 1.75mL of Glen-Pak RNA quenching buffer (Glen Research) was added to it. It was mixed well and the RNA immediately loaded on the Glen-Pak RNA purification cartridge. Following the Glen-Pak RNA purification protocol provided by the manufacturers, the collected DNA product was freeze dried. The purified RNA probe was dissolved in RNase-free water and quantified by determining the UV absorption at 260nm, after which the probe was split into several tubes to avoid contamination and stored in the freezer at -20°C for future experiments.

Table S1. Sequences of oligonucleotides synthesized.

Name	Sequence
RNA template	5'-AGC GAC UCU GAG GAG GAA CAA GAA GAU GAG GAA GAA AUC GAU GUU GUU UCU GUG GAA AAG AGG CAG GCU CCU GGC AAA-3'
DNA template	5'-AGC GAC TCT GAG GAG GAA CAA GAA GAT GAG GAA GAA ATC GAT GTT GTT TCT GTG GAA AAG AGG CAG GCT CCT GGC AAA-3'
Forward primer for cMyc	5'-AGC GAC TCT GAG GAG GAA CAA G-3'
Reverse primer for cMyc	5'-Acrydited- GCC AGG AGC CTG CCT CTT T-3'
Forward primer for EpCAM	5'-GCT CAG GAA GAA TGT GTC TGT GA-3'
Reverse primer for EpCAM	5'-Acrydited- GCA GCC AGC TTT GAG CAA ATG ACA-3'

The Efficiency of RT-PCR in Different Concentrations of Agarose Solutions. To study whether agarose would inhibit RT-PCR, a real-time quantitative reverse transcription PCR assay was performed (n=3) using a StepOne™ Real-Time PCR System (Applied Biosystems). RT-PCR was performed in different concentrations of agarose solutions. RT-PCR reaction mixture prepared with the final concentrations were as follows: 1×RT-PCR buffer, 0.4 mM of dNTP Mixture, 1.2 unit of RNasin, 0.375 unit of Hotmaster Taq polymerase, 1.2 unit of Quant RTase, 0.5μM of forward primer (5′-AGC GAC TCT GAG GAG GAA CAA G-3′), 0.5μM of ungrafted reverse primer (5′- GCC AGG AGC CTG CCT CTT T-3′), and 1× SYBR. The concentrations of agarose in five different samples were: 0%, 1%, 2%, 3%, 4% separately. The thermal cycling conditions were as follows: 50°C for 30 min, 94°C for 3 min (initial denaturation), 30 cycles of 94°C for 30 sec, 54°C for 30 sec, and 65°C for 30 sec, followed by a single final extension for 5 min at 65°C. As the result shows, the efficiency of 2% agarose solution was as high as in the 0% agarose solution (Fig. S1) while the mechanical strength of agarose beads was very high. Therefore 2% agarose solution was chosen as the optimized concentration in droplets to achieve droplet RT-PCR.

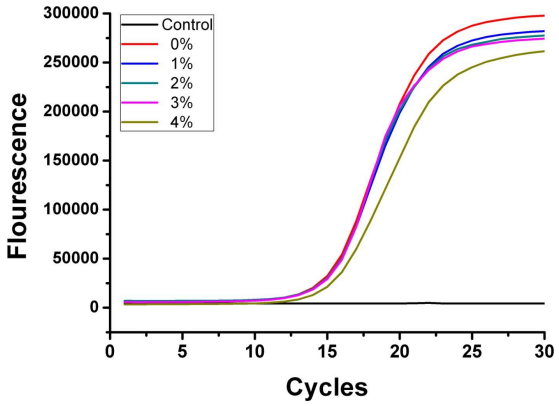

Figure S1. Real-time quantitative reverse transcription PCR results of amplicons in different concentration agarose solution.

Quantification of Droplet RT-PCR Efficiency. In order to further determine the performance efficiency of the RT-PCR in agarose droplets using the grafted primer, a real-time quantitative PCR assay was performed (n=3) using a StepOne™ Real-Time PCR System (Applied Biosystems). The reaction mixture was prepared according to the following final concentrations: 1× PCR buffer, 0.5μM of forward primer (5′-AGC GAC TCT GAG GAG GAA CAA G-3′), 0.5μM of ungrafted reverse primer (5′-AGC GAC TCT GAG GAG GAA CAA G-3′), 0.375μM of dNTPs, 0.5 unit of easy Taq polymerase and 1× SYBR Green. The copies of template DNA in five standard samples were 6.0E+06, 6.0E+07, 6.0E+08, 6.0E+09, 6.0E+10 separately. For unknown samples, 100 beads, produced from 29 cycles of agarose droplet emulsion RT-PCR with initial template concentration of 1.5 RNA copies/droplet, were added. As shown in Fig.S2 (B), as the cycle number increased, the amount of amplified product increased at different rates. It was found that the higher the initial concentration of DNA template, the lower the required cycle threshold number (Ct). A linear relationship between the Ct value and the initial DNA template concentration was observed (Fig.S2 A). The average numbers of amplicons (N) in each microbead after 29 cycles of agarose droplet emulsion RT-PCR from RNA concentration of 1.5 copies RNA/droplet were about 3.89E+7. Correspondingly, the RT-PCR efficiency ($E= (N/1.5)^{1/29}-1)*100\%$) in agarose droplets with a template concentration of 1.5 copies/bead, as calculated was 80.1%.

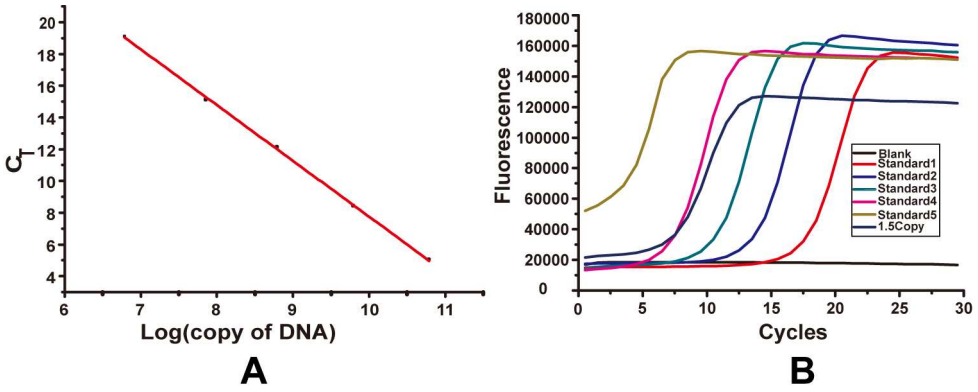

Figure S2. Q-PCR analysis results of amplicons in agarose beads amplified from 1.5 copy of RNA/droplet.

Quantification of EpCAM RNA expression level of Kato III cell and MDA-MB-231 cell. In order to evaluate whether EpCAM RNA translation levels in the Kato III cell and in the MDA-MB-231 cell are different, a quantification reverse translation PCR was conducted using a StepOne™ Real-Time PCR System. The reaction solution was prepared according to the following final concentrations: 1×RT-PCR buffer, 0.4 mM of dNTP Mixture, 1.2 unit of RNasin, 0.375 unit of Hotmaster Taq polymerase, 1.2 unit of Quant RTase, 0.5% Triton X-100 surfactant, 0.5μM of forward primer (5′-GCT CAG GAA GAA TGT GTC TGT GA-3′)**,** 0.5μM of ungrafted reverse primer (5′-GCA GCC AGC TTT GAG CAA ATG ACA-3′), and 1× SYBR. The copies for two different cells RT-PCR in three samples were 1E+04, 1E+03, 1E+02. The thermal cycling conditions were as follows: 50°C for 30 min, 94°C for 5 min (initial denaturation), 30 cycles of 94°C for 30 sec, 54°C for 30 sec, and 65°C for 30 sec, followed by a single final extension for 5 min at 65°C. As shown in Fig.S3, the cycle threshold number of the Kato III cells was lower than that of the MDA-MB-231 cells after performing RT-PCR with a similar number of cells. This confirms that the RNA transcription level in Kato III cells is higher compared with that of MDA-MB-231 cells.

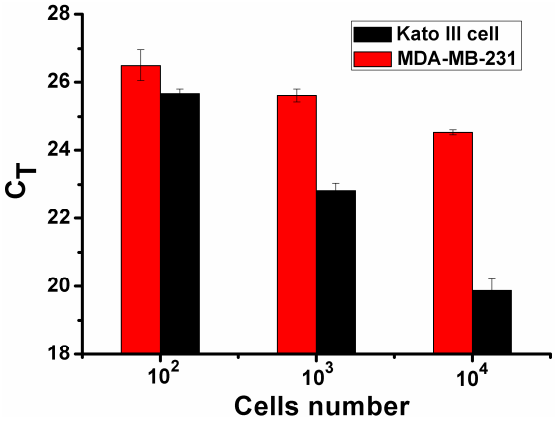

Figure S3. Q-PCR analysis results of of EpCAM RNA translation level of Kato III cells and MDA-MB-231 cells