Electronic Supplementary Information

A universal real-time PCR assay for rapid microRNAs quantification via the enhancement of base-stacking hybridization

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Reagents. TransStart Taq DNA polymerase was purchased from TransGen Biotech (Beijing, China). RNase Inhibitor was purchased from Thermo Fisher Scientific Co., Ltd. SYBR Green I (10,000× stock solution in dimethyl sulfoxide) was purchased from Life Technologies (AB & Invitrogen, Carlsbad, USA). HPLC-purified miRNAs, dNTPs, qRT-PCR kit and diethylpyrocarbonate (DEPC)-treated water were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). PAGE-purified DNA oligonucleotides were customized from Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). In order to create and maintain an RNase-free environment, the solutions were treated with 0.1% DEPC and autoclaved. The tips and tubes are RNase-free and do not require pretreatment to inactivate RNases. The buffers for cell experiments were prepared using distilled water purified by a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA) with an electrical resistance of 18.2 MΩ·cm. The buffers used in cellular extracts were prepared as follows. Buffer A was 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT. Buffer B was 20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM DTT. Buffer C was 20 mM HEPES (pH 7.9), 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT.

Table S1 The sequence information of miRNAs and DNA oligonucleotides used in this work.

Name	Sequence* (5′-3′)
let-7a	UGAGGUAGUAGGUUGUAUAGUU
let-7b	UGAGGUAGUAGGUUGU<u>G</u>U<u>G</u>GUU
let-7d	<u>A</u>GAGGUAGUAGGUUG<u>C</u>AUAGUU
let-7e	UGAGGUAG<u>G</u>AGGUUGUAUAGU
miR-141	U<u>A</u>A<u>CACU</u>GU<u>CU</u>G<u>G</u>U<u>AA</u>A<u>GAUGG</u>
miR-21	U<u>AGCU</u>UA<u>UC</u>AG<u>AC</u>UG<u>AUGUUGA</u>
miR-200b	U<u>AAUACU</u>G<u>CCU</u>G<u>G</u>U<u>AAUGAUGA</u>
template	GGCTAAGACAGATGCTCTTTGCCAACAGGCCACAGAATTCCTACA CTCAAAGTCGTACTGAACTATACAACCTACTACCTCATCGCACT
reverse primer	GGCTAAGACAGATGCTC
forward primer(P-4+7)	CCACGAGGCGA
forward primer(P-4+9)	CCACAAGAGGCGA
forward primer(P-4+11)	GTCCACAAGAGGCGA
forward primer(P-5+7)	GCGACGATGCGA
forward primer(P-5+9)	ATGCGACGATGCGA
forward primer(P-5+11)	AGATGCTACGATGCGA
forward primer(P-6+7)	ACGGAAGGTGCGA
forward primer(P-6+9)	CTACGGAAGGTGCGA
forward primer(P-6+11)	AACTACAGAAGGTGCGA
forward primer(P-7+7)	ACGGAAGAGTGCGA
forward primer(P-7+9)	ATACGGAAGAGTGCGA
forward primer(P-7+11)	TTCAACAAGAGAGTGCGA

* Underlined characters represent the different bases in the tested miRNAs compared with let-7a.

Real-Time PCR Assay. Real-time PCR experiment was performed on Bio-Rad CFX 96 Real-Time PCR instrument (BIO-RAD, USA). The PCR reaction mixture was prepared in a volume of 20 μL, including 0.5 U TransStart Taq DNA Polymerase, 1×TransStart Taq buffer (50 mM Tris-HCl, pH 9.0, 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$), 0.2 mM dNTPs, 600 nM forward primer, 600 nM reverse primer, 0.4×SYBR Green I, 5 nM template, 0.8 U/μL RNase Inhibitor, miRNA and DEPC-treated water. The PCR reaction was carried out by using hot start of 94 ºC for 30 s, followed by 30 cycles of 94 ºC for 5 s and 60 ºC for 30 s.

Cell Culture. Human prostate carcinoma cell lines (DU145), human hepatocellular carcinoma cell lines (BEL-7404), human cervical cancer cell lines (HeLa) and human breast cancer cell lines (MDA-MB231) were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). DU145 cells, BEL-7404 cells, HeLa cells and MDA-MB231 cells

were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin at 37ºC in a humidified 5% CO_2 incubator, respectively. The cells were grown with fresh medium at 50 mm glass-bottom dishes. Then the cells were harvested by trypsinization and washed with fresh medium for three times and suspended in fresh medium for following studies.

Preparation of cellular extracts. The cellular extracts were prepared according to the reported method with minor modification.[1] Briefly, 10^7 cells were washed once with phosphate-buffered saline and twice with Buffer A. The cell pellet was suspended in Buffer B/0.1% Nonidet P-40 (20 μL per 10^7 cells). After incubating for 15 min on ice, the lyzed cellular suspension was briefly mixed on a vortex and centrifugated for 10 min at 4 ºC. Then supernatant was diluted with 80 μL per 10^7 cells of Buffer C and stored at -80 ºC.

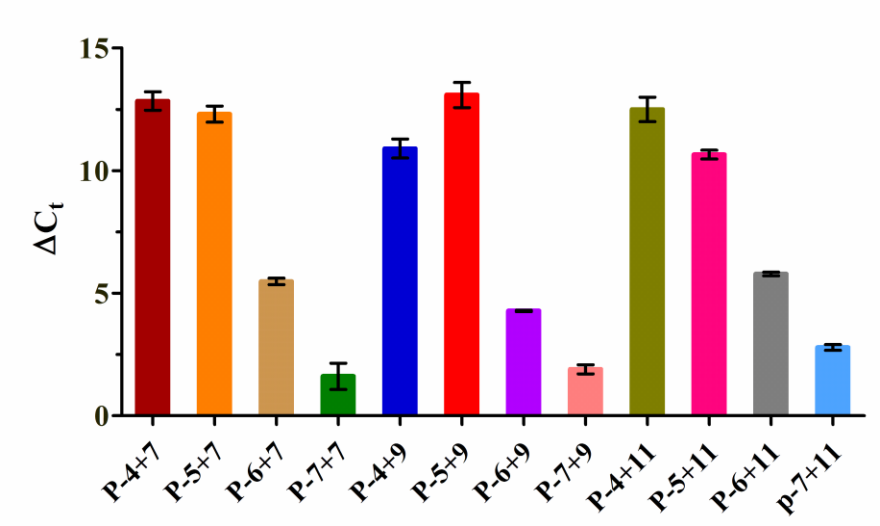

Fig. S1 Comparison of ΔC_t values among the different forward primers. The reaction were performed with 0.05 U/μL TransStart Taq DNA Polymerase, 1×TransStart Taq buffer, 0.2 mM dNTPs, 600 nM forward primer (P-4+7, P-5+7, P-6+7, P-7+7, P-4+9, P-5+9, P-6+9, P-7+9, P-4+11, P-5+11, P-6+11, P-7+11, respectively), 600 nM reverse primer, 0.4×SYBR Green I, 10 nM template, 1 nM let-7a, 16 U RNase Inhibitor, and DEPC-treated water.

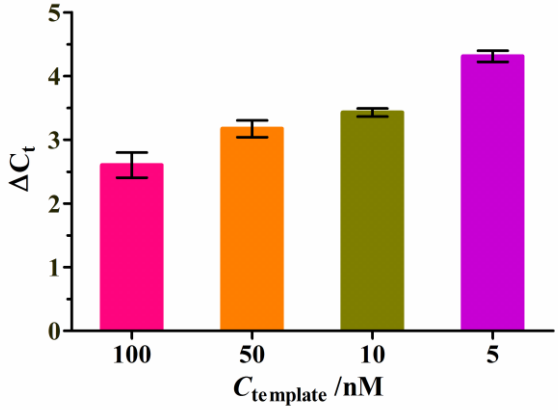

Fig. S2 The effect of the amount of the template on ΔC_t value. The reaction were performed with 0.05 U/μL TransStart Taq DNA Polymerase, 1×TransStart Taq buffer, 0.2 mM dNTPs, 600 nM forward primer, 600 nM reverse primer, 0.4×SYBR Green I, template (100 nM, 50 nM, 10 nM, and 5 nM, respectively), 100 pM let-7a, 16 U RNase Inhibitor, and DEPC-treated water.

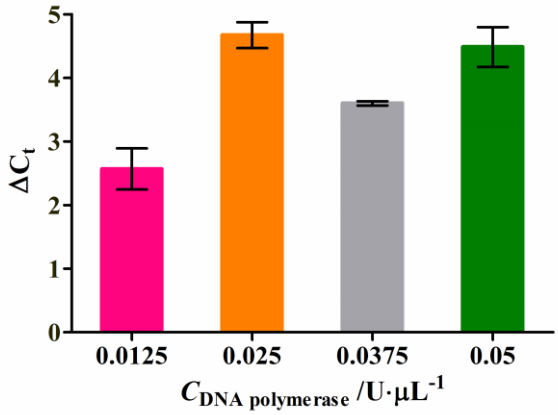

Fig. S3 Optimization of the amount of DNA polymerase. The reaction were performed with TransStart Taq DNA Polymerase (0.0125 U/μL, 0.025 U/μL, 0.0375 U/μL, and 0.05 U/μL, respectively), 1×TransStart Taq buffer, 0.2 mM dNTPs, 600 nM forward primer, 600 nM reverse primer, 0.4×SYBR Green I, 5 nM template, 100 pM let-7a, 16 U RNase Inhibitor, and DEPC-treated water.

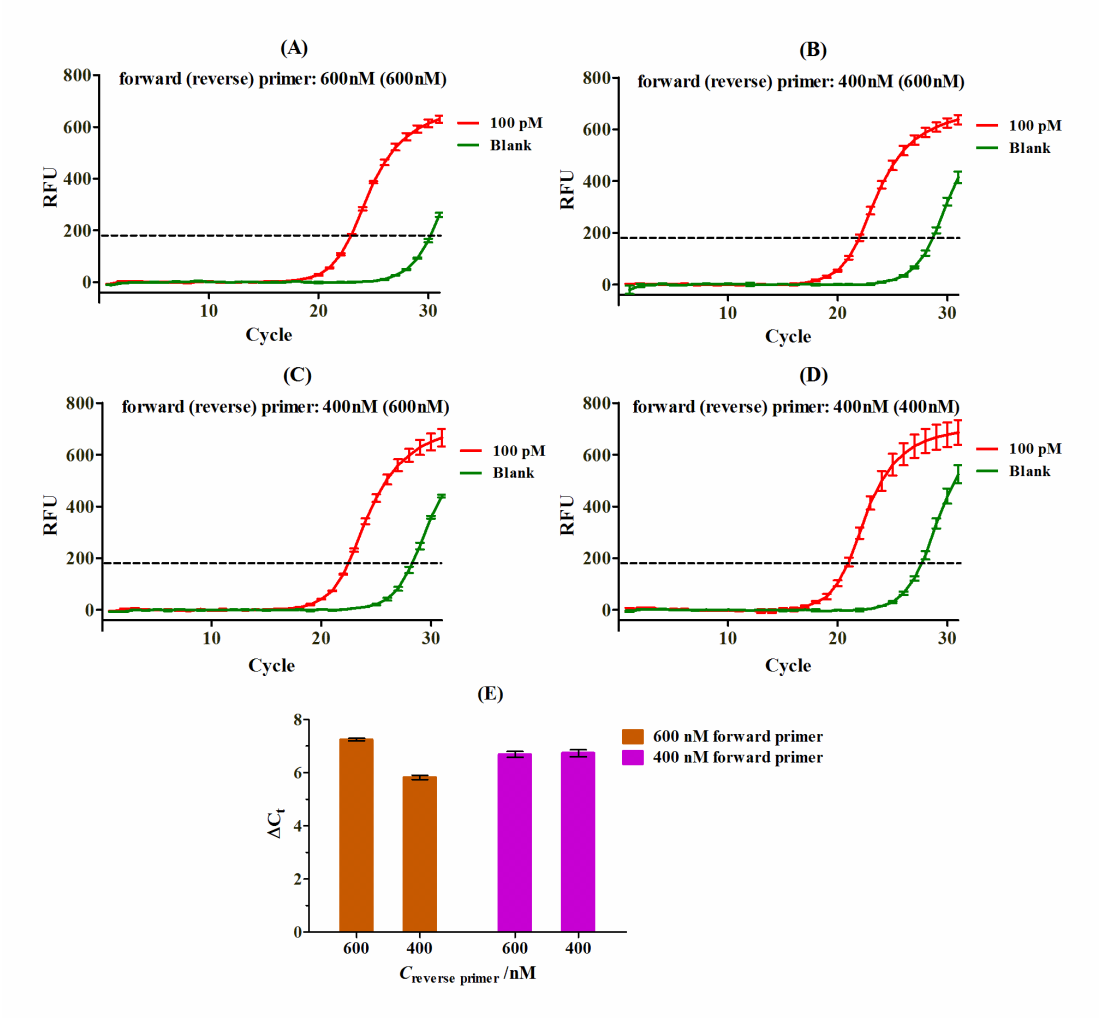

Fig. S4 Optimization of the concentrations of the forward primer and the reverse primer. The reaction were performed with 0.025 U/μL TransStart Taq DNA Polymerase, 1×TransStart Taq buffer, 0.2 mM dNTPs, forward primer (400 nM and 600 nM), reverse primer (400 nM and 600 nM), 0.4×SYBR Green I, 5 nM template, 100 pM let-7a, 16 U RNase Inhibitor, and DEPC-treated water.

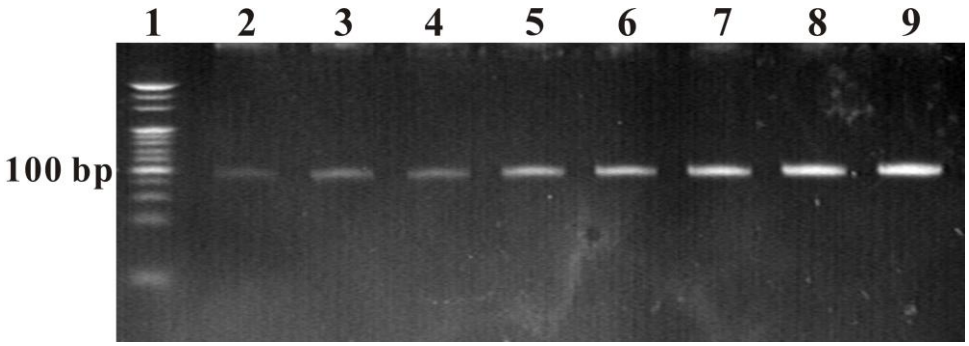

Fig. S5 Non-denaturing polyacrylamide gel electrophoresis verification of the products from the base-stacking hybridization assisted polymerase chain reaction in the prescence of let-7a with different concentrations. Lane **1**: DNA marker, lane **2**: 0, lane **3**: 500 fM, lane **4**: 5 pM, lane **5**: 50 pM, lane **6**: 100 pM, lane **7**: 500 pM, lane **8**: 1 nM, lane **9**: 10 nM.

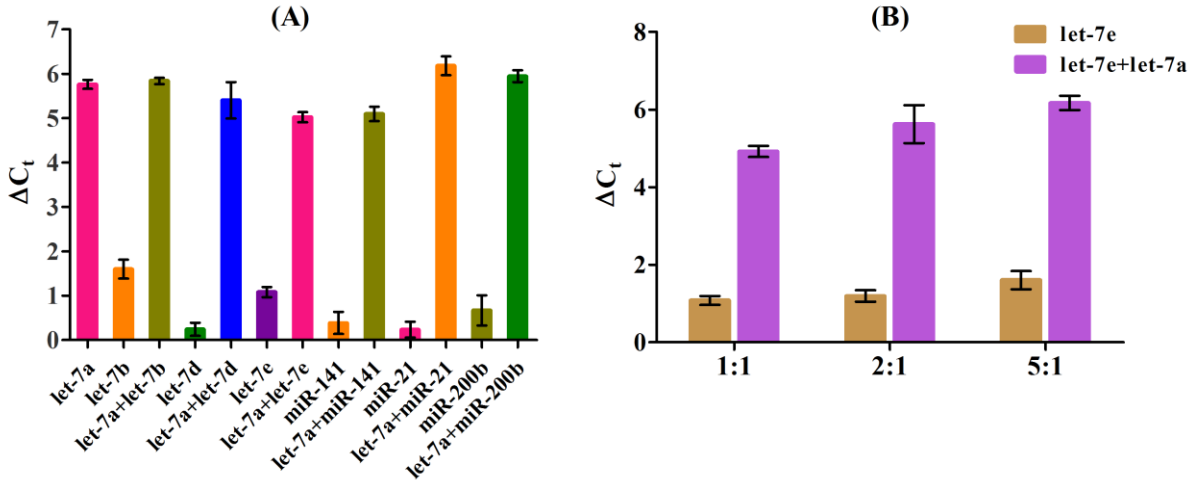

Fig. S6 Selectivity investigation of the proposed method using different miRNAs and admixture. (A) Bar representing ΔC_t values from the different inputs of let-7a, let-7b, let-7d, let-7e, miR-141, miR-21, miR-200b, a mixture of let-7a and let-7b, a mixture of let-7a and let-7d, a mixture of let-7a and let-7e, a mixture of let-7a and miR-14, a mixture of let-7a and miR-21, and a mixture of let-7a and miR-200b. The concentrations of the tested miRNAs were all 100 pM. (B) Bar representing ΔC_t values from the let-7e with different concentrations (100 pM, 200 pM, and 500 pM), and the mixtures of let-7e and let-7a with different concentration ratios of let-7e and let-7a in 1:1, 2:1, and 5:1. The let-7a concentration was 100 pM in the mixtures.

Reference

1 L. Osborn, S. Kunkel, G. J. Nabel, *Proc. Natl. Acad. Sci. U S A* 1989, **86**, 2336.