

Liposomes-encoded Magnetic bead Initiated by Padlock Exponential Rolling Circle Amplification for Portable and Accurate Quantification of MicroRNA

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EXPERIMENTAL SECTION

Chemicals and Materials

The HPLC-purified miRNAs and RT-PCR kit were obtained from Takara Biotechnology Co. Ltd. (Dalian, China). Target miRNA-21: 5'-UAG CUU AUC AG A CUG AUG UUG A-3', miRNA-122: UGG AGU GUG ACA AUG GUG UUU G-3', 1-mismatch: 5'-UAG AUU AUC AG A CUG AUG UUG A-3', 2-mismatch: 5'-UAG AUU AUC AG A CUG ACG UUG A-3', Other oligonucleotide sequences were synthesized and purified by HPLC at Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). The sequences are as follows: padlock probe: 5'-phosphate-CTG ATA AGC TA CCTCAGC CAA ACC ATA ATC CCT ACA A CCTCAGC CAA ACC ATA ATC CCT ACA ACCTCAGC TCA ACA TCA GT-3', the biotinylated capture probe (cDNA): 3'-biotin-A₂₀-CG GTT TGG TAT TA-5', the amino-terminated reporter probe (pDNA): 3'- NH₂-A₂₀-GGGT TGT T GGA GT-5'. Phi29 DNA polymerase, T4 RNA ligase 2, dNTP, and Nb.BbvCI were obtained from New England Biolabs (Ipswich, Massachusetts). 1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethyleneglycol)-2000] (ammonium salt) (DSPE-PEG-COOH) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Streptavidin-magnetic beads (MNBs) (830 nm diameter) were obtained from Bangs Laboratories Inc. (Fishers, IN). Glucoamylase and amylose were purchased from Sigma-Aldrich. Other chemicals (analytical grade) were obtained from standard reagent suppliers.

Instruments

The transmission electron microscopic (TEM) images were performed on a JEM-2100 transmission electron microscope (JEOL), operating at 200 kV with an ultrahigh-resolution pole piece and providing a point resolution of 2 Å. Atomic force microscopy (AFM) was performed on a Bruker Multimode 8 atomic force microscope. RT-PCR was performed on a LightCycler480II. Hydrodynamic dynamic radius (R_H) and zeta-potential (ξ) of the glucoamylase-encapsulated liposome (GEL) were

measured by Malvern mastersizer 2000. The glucose signal was recorded by glucose meter.

Preparation of the glucoamylase-encapsulated liposome (GEL)

The preparation of glucoamylase-encapsulated liposome (GEL) was similar to our previous work with minor modifications.⁴² Briefly, three lipids, DSPC, β -cholestanol (Chol), and DSPE-PEG-COOH with a molar ratio of 70:10:20, were added into the flask with 3 mL chloroform, and mixed uniformly at 35 - 45 °C. Then, the chloroform was evaporated under vacuum for 30 min, and the resultant thin film adhering to the inside wall of flask was flushed with argon stream for 2 h to remove residual traces of organic solvent. Subsequently, the dried lipid film was rehydrated in 5 mL 1 mg/mL glucoamylase solution, and vortexed for 30 min in a bath sonicator (35 °C). The resulting solution was extruded 11 times through a polycarbonate membrane with 100 nm pores (Nucleopore). Finally, untrapped glucoamylases were removed by using gel filtration through a Sephadex G-100 column. The collected fraction of GEL was stored at 4 °C until use.

Preparation of cDNA-modified magnetic beads and pDNA-functionalized GEL

The carboxyl-functionalized GEL was first activated using 25 μ L 0.1 mmol EDC and 25 μ L 25 μ mol NHS coupling agents for 60 min reaction. Then, 20 μ L 24 nmol amino-terminated pDNA was added, and incubated for 2 h at room temperature with gentle shaking. Finally, the as-obtained pDNA-GEL was purified by centrifugation at 10000 rpm for 10 min to remove the unreacted pDNA as well as the coupling agents.

50 μ L streptavidin-functionalized magnetic beads (MNBs) (1 mg/mL) were first rinsed with PBS for three times. Then, the MNBs were resuspended in 200 μ L PBS buffer (pH 7.0). Subsequently, 25 nmol biotinylated cDNA was added, and incubated at room temperature for 2 h with gentle shaking. The resultant cDNA-MNBs were then rinsed with PBS-T and PBS for three times, respectively. Finally, the resultant cDNA-MNBs rinsed were resuspended in 200 μ L PBS buffer, and stored at 4 °C.

Preparation of P-ERCA products

The padlock probe ligation reaction was performed in 50 μL of reaction mixture containing 1 \times ligation buffer (50 mM Tris-HCl, 400 mM ATP, 2 mM MgCl_2 , 10 mM DTT, pH 7.5), 2 U T4 RNA ligase 2, 0.8 U RNase inhibitor, 150 nM padlock probe and different concentrations of miRNAs. First, the padlock probe and miRNA mixture was denatured at 65 $^\circ\text{C}$ for 3 min, and cooled slowly to room temperature. Then, T4 RNA ligase 2 and the ligation buffer were added into the mixture, and incubated at 37 $^\circ\text{C}$ for 2 h. Afterward, 5 U Phi29 DNA polymerase, 500 μM dNTP, 10 \times polymerase reaction buffer and 3 U Nb.BbvCI were added into 50 μL of ligation reaction products to form a 60 μL mixture. Finally, the mixture was incubated for 6 h at 30 $^\circ\text{C}$ for obtaining the products trigger DNA (t-DNA). The obtained products were stored at 4 $^\circ\text{C}$.

Liposomes-encoded magnetic bead strategy Assay procedure

To detect miRNA-21, 40 μL mixture containing cDNA-MNBs (0.25 mg/mL) and pDNA-GEL (1.5×10^{11} liposomes/mL) was first incubated with the trigger DNA (t-DNA) prepared by P-ERCA at 37 $^\circ\text{C}$ for 60 min. Then, the unreacted GEL was removed with magnetic separation, followed by washing the resultant liposome-encoded magnetic bead (MNBs-cDNA/t-DNA/pDNA-GEL) for three times with PBS. Next, 10 μL 1% Triton X-100 and 2.5 $\mu\text{g}/\mu\text{L}$ substrate amylose were added into the resultant liposome-encoded magnetic bead, and maintained for 40 min to lyse the liposomes for liberating the encapsulated glucoamylase. The released glucoamylase further catalyze the hydrolysis of amylose, producing a large amount of glucose. Finally, the glucose signal of the as-obtained solution was measured by a mini commercial GM.

Cell culture and miRNA extraction

Human breast cancer cell lines (MCF-7 and MDA-MB-231) and non-tumorigenic MCF-10A were obtained from American Type Culture Collection (Manassas, VA). The cell lines were cultured in DMEM supplemented with 10% fetal

bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The cell density was determined by a hemacytometer. RNA was extracted using Direct-zol™ kit (Zymo Research, USA).

Determination of miRNA-21 in human breast cancer cells

MiRNAs were extracted from human breast adenocarcinoma cells (MCF-7 and MDA-MB-231) cells and epithelial nontumorigenic (MCF-10A cells) cells by using a commercial total-RNA extracting kit according to the manufacturer's procedures. The cell lines are processed with cell counting before extraction. The RNA concentration was determined by the UV-vis absorption at 260 nm. The sample of miRNA-21 in these cells was diluted, and then analyzed with the proposed assays.

Results and discussion

Characterization of glucoamylase-encapsulated Liposome (GEL)

The diameter (d) and surface charge zeta-potential (ξ) of GEL were also investigated. An average hydrodynamic diameter of approximately 122 ± 22 nm in size was showed (**Fig. S1**). And, a reduced surface charge zeta-potential (ξ) (-19.5 ± 2.6 mV) was given (**Table S1**). The zeta-potential measured indicate that the GEL is highly dispersible in aqueous solution.

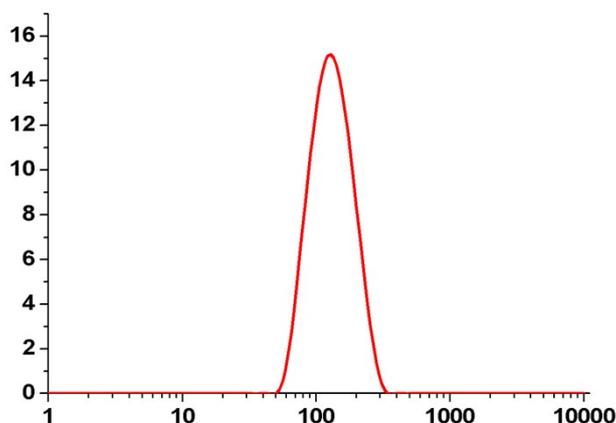


Fig. S1. Diameter distribution of glucoamylase-encapsulated liposome (GEL).

Table S1. Characterizations of glucoamylase-encapsulated liposome (GEL).

Sample	mean diameter (nm) ^a	Zeta potential (mV) ^a
glucoamylase-encapsulated liposome (GEL)	122 ± 22 nm	-19.5 ± 2.6 mV

^a Mean ± standard deviation, n=3; Mean diameter is the hydrodynamic diameter; Zeta potential indicates the average surface charge.

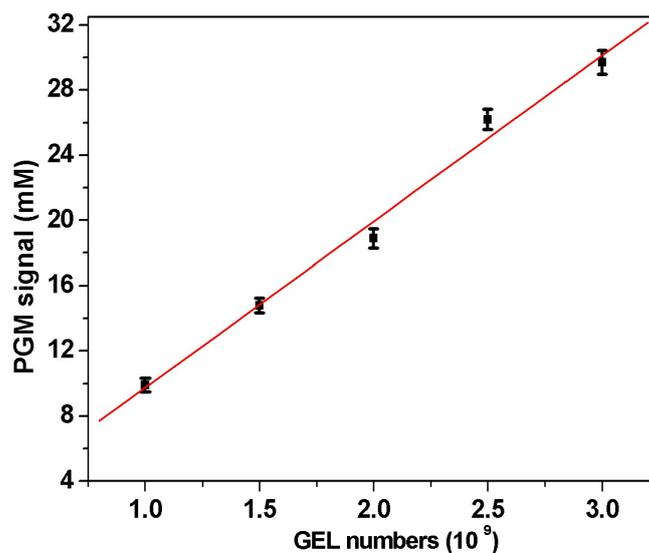


Fig. S2 PGM signal intensity versus the number of glucoamylase-encapsulated liposomes (GEL).

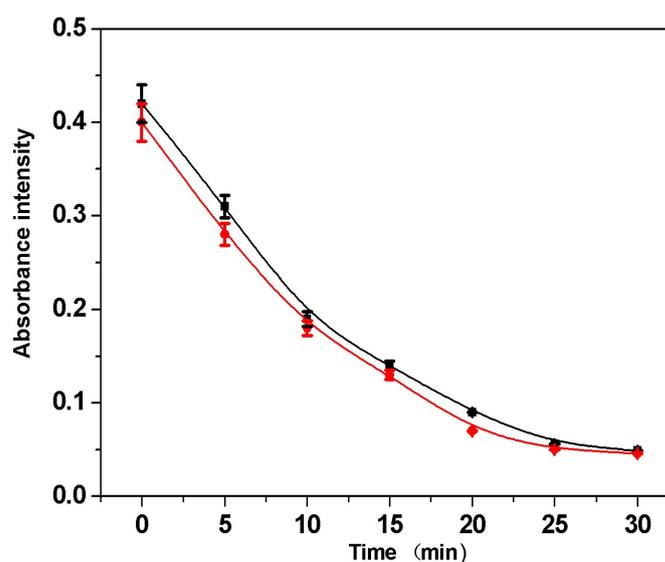


Fig. S3 Comparison of glucoamylase activity in free solution (black) and GEL solution (red) after complete release by monitoring the absorbance at 547 nm of the amylose-KI/I₂ solution. The concentrations of glucoamylase and amylose were the

same under the two conditions.

Optimization of assay conditions

In the sensor strategy, P-ERCA play important role in the formation of liposome-encoded magnetic bead. To achieve the optimal sensing performance, P-ERCA experimental parameters such as concentration of padlock probe, dNTP substrates, Phi29 DNA polymerase and Nb.BbvCI were investigated in this study. The concentrations of dNTP, Phi29 DNA polymerase and Nb.BbvCI are the key factors affecting the trigger DNA product by P-ERCA. As shown in **Fig. S4A-D**, we can easily see that the PGM signal intensity can reach a steady value when concentrations of padlock probe, dNTP, Phi29 DNA polymerase and Nb.BbvCI were 150 nM, 500 μ M, 5 U and 3 U, respectively. Therefore, 150 nM padlock probe, 500 μ M dNTP, 5 U phi29 DNA polymerase and 3 U of Nb.BbvCI were used for the P-ERCA in the sensing performance.

Additionally, the preparation of liposome-encoded magnetic bead is a critical step for the assay of miRNA-21. Several experimental parameters affecting the preparation of liposomes-encoded magnetic bead were optimized such as the amounts of cDNA for magnetic beads modification and the incubation time of trigger DNA hybridization. As shown in **Fig. S5A**, the glucose signal intensity increased gradually with the cDNA amounts from 10 nmol to 40 nmol. It could be observed that the glucose signal intensity is the highest when the cDNA amount was 25 nmol, and this amount was selected to modify the magnetic bead. The higher concentration of cDNA can increase the liposome density encoded on the MNBs, but it may restrict the hybridization owing to the steric hindrance effect. In addition, the incubation time of trigger DNA was also investigated (**Fig. S5B**) and the experimental results indicated that 60 min could provide maximum glucose signal intensity for the sensing system. Therefore, the incubation time of trigger DNA was chosen with 60 min as the optimized incubation time.

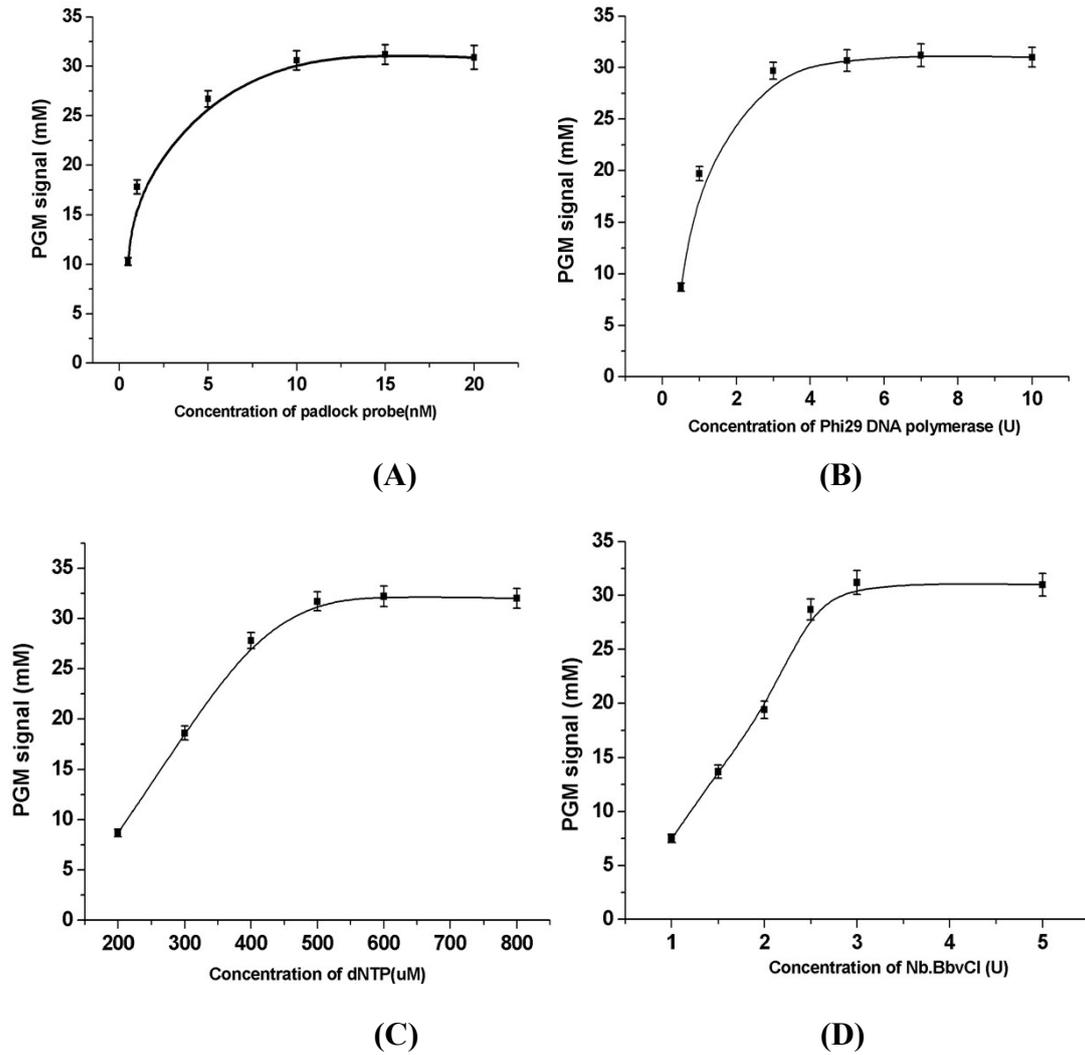
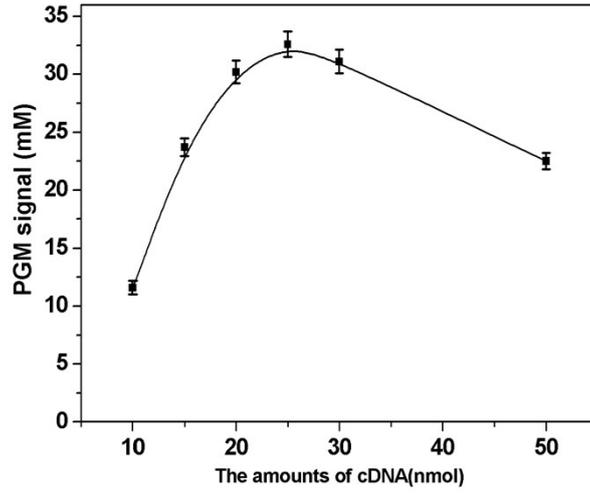
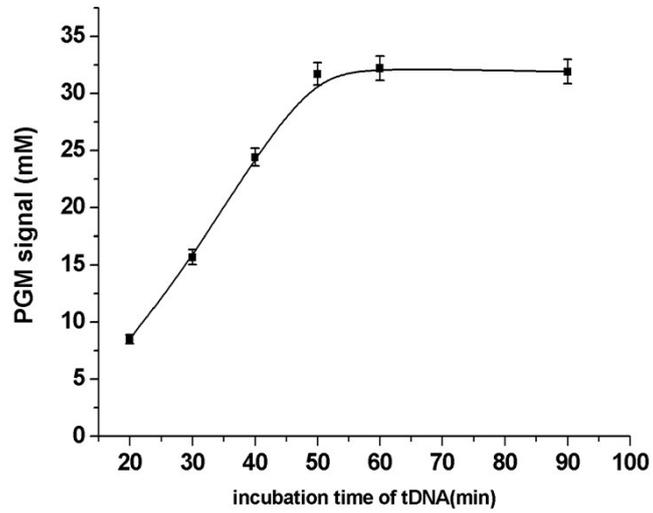


Fig. S4 Optimization of concentration of (A) padlock probe, (B) Phi29 DNA polymerase, (C) dNTP, and (D) Nb.BbvCI. The assays were carried out in the reaction buffer containing 1pM miRNA.



(A)



(B)

Fig. S5 Optimization of (A) concentration of cDNA probe, (B) incubation time of tDNA. The assays were carried out in the reaction buffer containing 1pM miRNA.

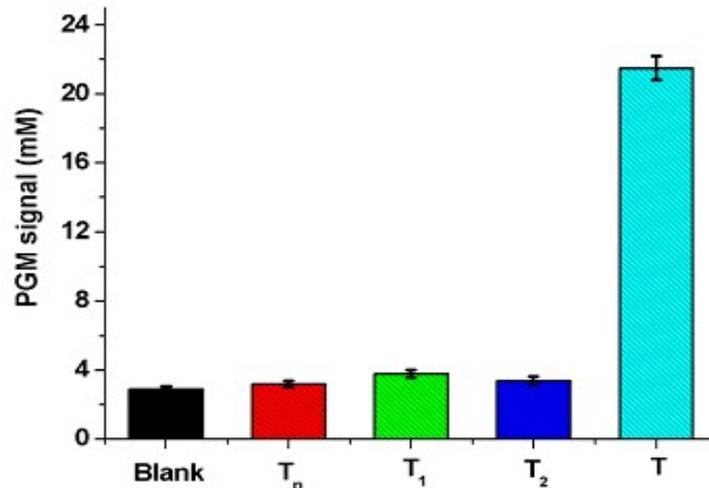


Fig. S6 Selectivity of the sensing system for different targets: blank; (T_n) miRNA-122 (1 pM); (T₁) one-base mismatched stand (1 pM); (T₂) two-base mismatched stand (1 pM); (T) miRNA-21 (100 fM). Error bars show the standard deviation of three experiments.

Procedure of RT-RCR

The reactions were performed using a Mir-X™ miRNA First-Strand Synthesis and SYBR®qRT-PCR kit according to manufacturer's instructions. Firstly, the reverse transcription of extracted miRNA to cDNA was conducted and analyzed with PCR. The following reagents including mRQ Buffer, RNA sample and mRQ Enzyme were mixed in an RNase-free 0.2 mL tube with a total volume of 10 μL. The tube was incubated at 37 °C for 1 h, then terminate at 85 °C for 5 min to inactivate the enzymes. 90 μL ddH₂O was subsequently added to make up a total volume of 100 μL. The resulted cDNAs were ready for the miRNA quantification. 25 μL PCR solution, containing cDNA, has-miRNA-21 specific primer and mRQ 3' primer, was followed the procedures for amplification: Denaturation: 95 °C for 10 sec; qPCR x 40 Cycles: 95 °C for 5 sec, 60 °C for 20 sec; Dissociation Curve: 95 °C for 60 sec, 55 °C for 30 sec, 95 °C for 30 sec.

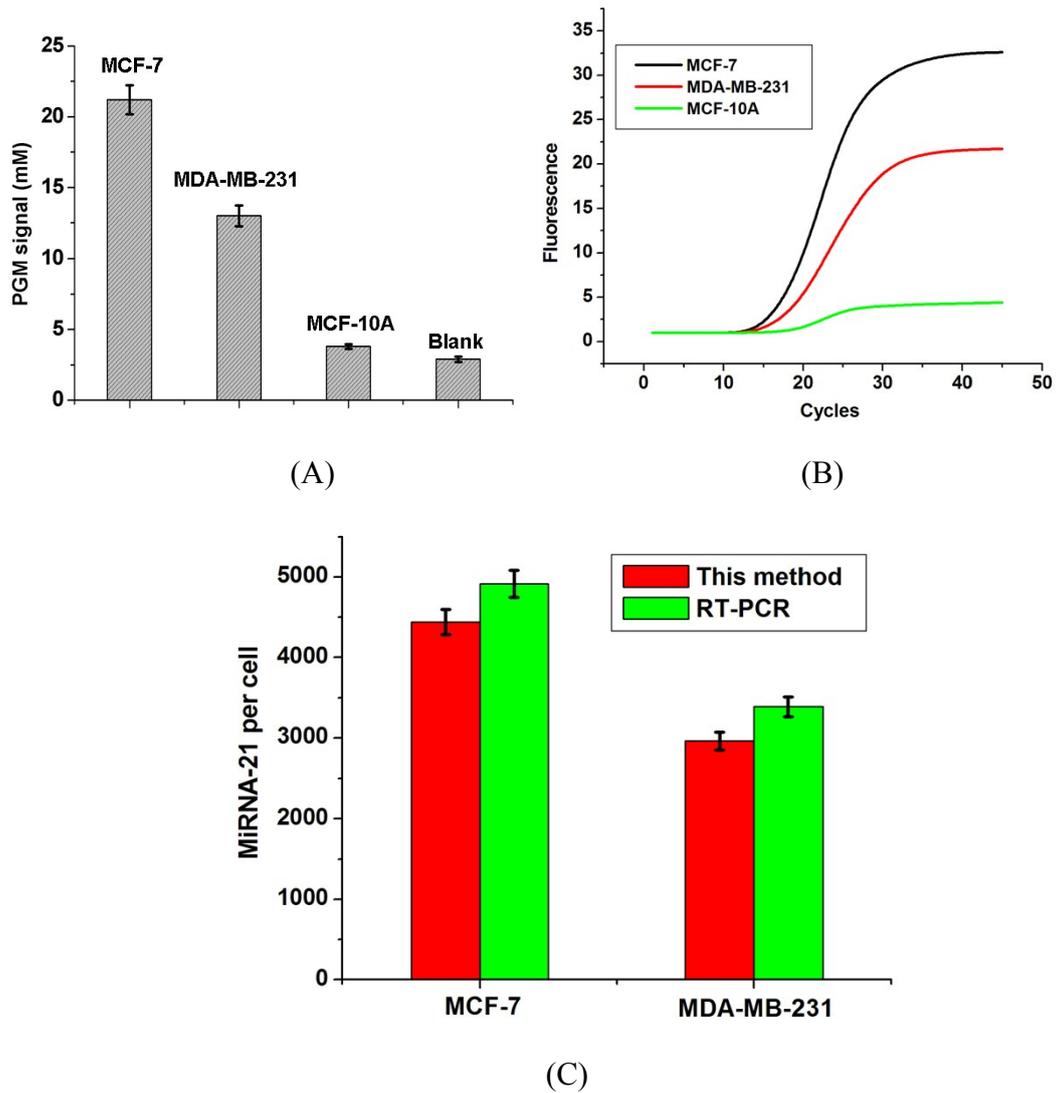


Fig. S7 (A) Signal quantification of the PGM based on liposome-encoded magnetic bead initiated by P-ERCA after incubating with miRNA-21 extracted from various cell lines. **(B)** Quantitative real-time fluorescence monitoring of the PCR amplification reaction triggered by miRNA-21 extracted from different kinds of cells. **(C)** Quantitative detection of miRNA-21 in each cell by the PGM based on liposome-encoded magnetic bead initiated by P-ERCA assay and RT-PCR

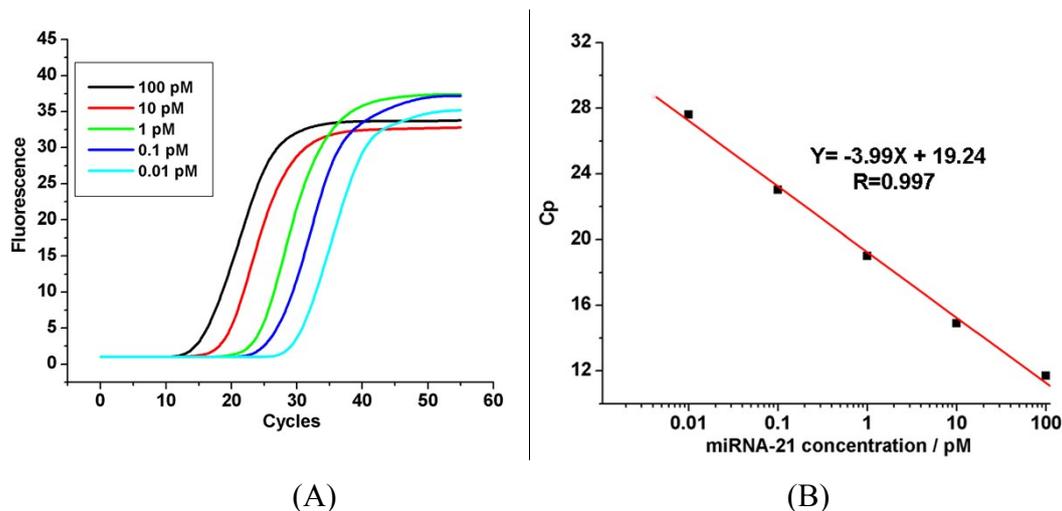


Fig. S8 RT-PCR is employed to validate the accuracy of the PGM assay based on liposome-encoded magnetic bead initiated by P-ERCA. (a) Quantitative real-time fluorescence response profiling of miRNA-21 at different concentrations by RT-PCR. (b) Plots of the C_p values versus the concentrations of miRNA-21, the linear range of RT-PCR is from 100 pM to 0.01 pM.

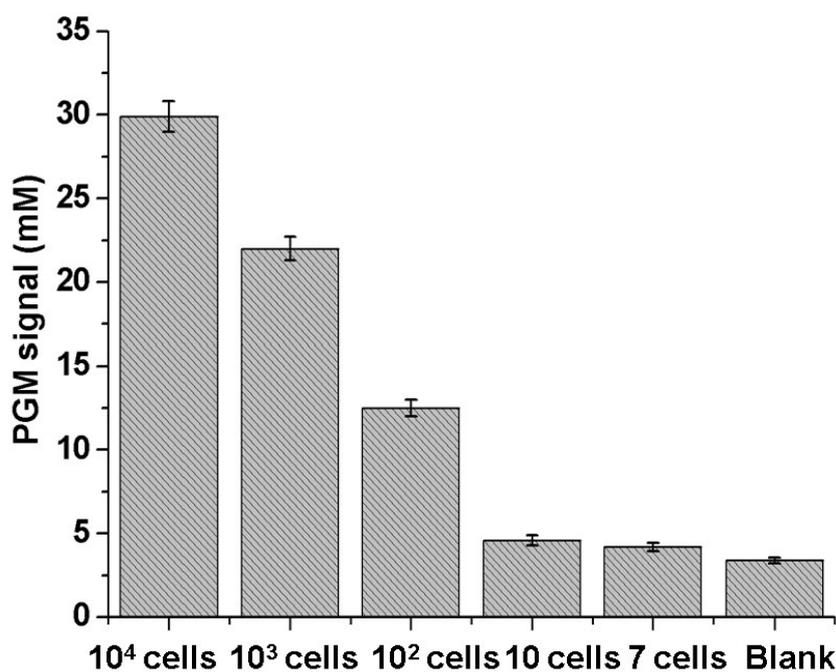


Fig. S9 Signal quantification of the PGM based on liposome-encoded magnetic bead initiated by P-ERCA in the presence of diluted miRNA-21 extracted from MDA-MB-231 cells from 7 to 10000 cells.