Electronic Supporting Information (ESI)

Dual amplification of bio-barcode and auto-cycling primer extension for highly sensitive detection of miRNA

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Experimental Section

Chemicals and Materials

SYBR Gold was purchased from Invitrogen (USA). Six times loading buffer was bought from TaKaRa Bio Inc. (Dalian, China). 30 K ultrafiltration centrifuge tubes were obtained from Oaco Bioreagent Co., Ltd (Changsha). All other reagents were analytically grade. All ultrapure water (≥18 MΩ, Milli-Q, Millipore) used throughout experiment was sterilized. All oligonucleotides were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China), and the sequences are listed in Table S1.

Apparatus

Centrifuge was carried out by Beckman Coulter Allegra 25R centrifuge (Brea, CA, USA). Gel imaging was performed on Azure C600 (America). All buffer pH measurements were performed on Orion 3 Star pH meter (Thermo Scientific, USA). All fluorescence spectra were obtained on an F-7000 fluorescence spectrometer (Hitachi, Japan).

The preparation of DNA probes

The DNA probes used in the dual amplification consisted of six oligonucleotide strands, namely copy-and-release hairpin (CRH), capture probe (CP), assistant probe (AP), FAM-labeled primer (FAM-DP), quencher-labeled primer (BHQ-DP), and DNA-19a. The six oligonucleotide strands were first centrifuged at 12000 rpm, 4°C for 5 min. CP was dissolved into 10 μM stock solution with magnetic bead washing buffer, and other strands was dissolved into 100 μM stock solution with 10 mM PBS solution or sterilized water. For the synthesis of DNA probes, FAM-DP and BHQ-DP were mixed together with concentrations of 1 μM and 1.5 μM, then annealed at 95°C for 5 min and slowly cooled to room temperature. The CRHs was annealed at 95°C for 5 min and slowly cooled to room temperature.

The preparation, functionalization, and characterization of gold nanoparticles (AuNPs)

The 13 nm AuNPs were synthesized using the standard sodium citrate reduction method.12 Before this, all glassware was cleaned in aqua regia (HCl: HNO3=3:1), rinsed fully with deionized water and then oven-dried. Next, 0.01% HAuCl4 (100 mL) was heated to boiling with vigorous stirring, followed by adding 3.5 mL trisodium citrate (1%) under stirring. The solution color turned from pale yellow to colorless and finally to burgundy. After cooling to room temperature, it was filtered by a 0.45 μm Millipore syringe filter. TEM and AFM experiments were performed to characterize the AuNPs.

Thiol-modified oligonucleotides and TCEP were added to 13 nm gold colloids at a concentration of 3 μM of oligonucleotides per 1 mL and shaken overnight. After 16 hours, Phosphate buffer (0.1 M; pH = 7.4) was added to the mixture to achieve a 0.01 M phosphate concentration, and aliquots of sodium chloride solution (2.0 M) were added to the mixture over an eight-hour period to achieve a final sodium chloride concentration of 0.3 M. The solution containing the functionalized particles was centrifuged (13,000 rpm, 20 min) and resuspended in phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Phosphate, 2.72 mM KCl, pH 7.4,

Hyclone) three times to produce the purified AuNPs used in all subsequent experiments. The UV-visible spectra of AuNP before and after modification in the range of 400-800 nm and the UV-visible spectra of the thiolated oligonucleotides in the supernatant solution before and after modification in the range of 220-400 nm were scanned. The particle size of AuNP before and after modification was also characterized by Malvern zeta-size meter.

The modification of magnetic microbeads (MMPs)

The MMPs were shaken on a vortex mixer for 20 s to disperse the beads, then washing buffer was added to wash thoroughly and repeat two times. Next, 10 μM CPs were added in the tubes and incubated on a rotary mixer for 30 min at room temperature. After magnetic separation, the supernatant was reserved for the quantification of UV absorption. The functionalized MMPs were washed three times with washing buffer before dispersing into PBS buffer and store at 4 °C. The surface density of the CPs on the MMPs can be calculated by measuring the UV absorption in the supernatant before and after the modification, according to the concentration of the magnetic beads.

Fluorescence experiments

To verify the auto-cycling primer extension reaction, the primer was labeled with FAM and a quencher strand labeled with BHQ1 was complemented with the primer. To form a stable hybrid complex, primer and the quencher strand(1:1.5) were heated at 95℃ for 5 min, and then cooled down to room temperature for the following use. Then, the mixture was added into CRH (the concentration of primer and CRH was 10:1), as well as Bst DNA Polymerase, dNTP, $MgSO_4$ and Bst reaction buffer in 10 mM PBS. After incubating for 4 h on a thermostatic metal shaker bath at 37℃ and then heated at 80℃ for 20 min, the fluorescence spectra were measured. With the addition of strand displacement reaction, the ratio of CRH and blocker was 1:1.2. After that, the following steps were as above, and the control experiment without target was performed under the same conditions. All fluorescence spectra were measured using a Hitachi F-7000 fluorescence spectrometer equipped with an aqueous thermostat (37℃, Amersham Biosciences, Sweden). The excitation wavelength was set at 488 nm, the emission spectra were collected from 510 to 650 nm, the scanning speed was 1200 nm/s, and the response time was 2 s. The excitation and emission slits were all 5 nm band-pass with a 700 V PMT voltage and a 0.2 × 1 cm^2 quartz cuvette. All experiments were repeated three times in parallel.

Sensitivity and specificity assays

According to the optimal experimental condition, in the auto-cycling primer extension amplification, reaction solution was incubated with various concentrations of DNA-19a (5 nM, 500 pM, 50 pM, 5 pM, 500 fM, 50 fM, 0 fM) in 10 mM PBS. The reaction solution was transferred to a colorimetric dish, and the temperature was kept at 37℃ for fluorescence time scanning. The scanning time range is set to 0-7200 s and other conditions of the experiments were the same as the above. For specificity assays, reaction solution was incubated with 5 nM miR-17-92 family (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, miR-92a) in 10 mM PBS. The reaction solution

was transferred to a colorimetric dish, and the temperature was kept at 37℃ for fluorescence time scanning. The scanning time range is set to 0-6000 s and other conditions of the experiments were the same as the above.

miRNA-19a detection in serum

First, the fetal bovine serum was treated with 0.2 U/μL RNase inhibitors. Next, the serum was centrifuged at 6000 rpm for 20 min and then filtrated through 30 K ultrafiltration centrifuge tubes to remove biomacromolecules. After that, various concentration of miR-19a was added into diluted human serum samples. Finally, the prepared samples were measured on the F-7000 fluorescence spectrometer after excitation at 488 nm and by measuring emission at 520 nm from 0 to 7200 s.

miRNA-19a detection in cell lysates

MCF-7 cells (breast cancer cell lines) and MCF-10a cells (breast epithelial cells) were purchased from the cell bank of the Chinese Academy of Science (Shanghai, China). First, the cells were washed three times by 4 mL of D-Hank's, mixed with 20 mL of fresh RPMI 1640 medium (supplemented with 10% FBS and 100 U/mL penicillin-streptomycin) and incubated at 37 °C for 24 h under a 5% CO2 atmosphere. The digested cells were lysed and ultrafitered to remove the residual cells at 24 000 rpm for 30 min by a centrifugation (CS150NX, Hitachi, Japan). The supernatant media were divided into two equal parts for the detection using this method and quantitative real-time polymerase chain reaction (RT-qPCR). Results of RT-qPCR were analyzed with an SG Fast qPCR master mix (2×) on an ABI Stepone plus RT-qPCR system. Relative intensity of miRNA-19a in MCF-7 cells was normalized by the results of RT-qPCR experiments in MCF-10a cells.

Supporting Tables:

Table S1. Oligonucleotide sequences used in this work

Name	Sequence (5'-3')
CRH	TGCGCATTTTTGCGCA**mGmG**GCCTTTTGGC**mCmC**TGCGCAAAAATGCGCA GTAGTGTAATTTTTTTTTT-SH
AP	HS-TTTTTTTTTTTTTTTTTTTTTTTTTTCAGTTTTGCA
CP	TAGATTTGCACATTTTTTTTTT-Biotin
FAM-DP	FAM-CTTGGTTTACGTGTTTTACACTAC
BHQ-DP	AACACGTAAACCAAG-BHQ1
FAM-CRH	FAM-TGCGCATTTTTGCGCAGGGCCTTTTGGCCCTGCGCAAAAATGCGCAG TAGTGTAATTTTTTTTTT-SH
FAM-AP	HS-TTTTTTTTTTTTTTTTTTTTTTTTTTCAGTTTTGCA-FAM
DNA-17	ACTGCTGAGCTAGCACTTCCCGA
DNA-18a	ACTGCCCTAAGTGCTCCTTCTGG
DNA-19a	TGTGCAAATCTATGCAAAACTGA
DNA-19b	TGTGCAAATCCATGCAAAACTGA
DNA-20a	ACTGCATTATGAGCACTTAAAG
DNA-92a	TATTGCACTTGTCCCGGCCTGT
miR-19a	UGUGCAAAUCUAUGCAAAACUGA

mG, mC stand for 2’ -O-methyl G and 2’ -O-methyl C

Supporting Figures:

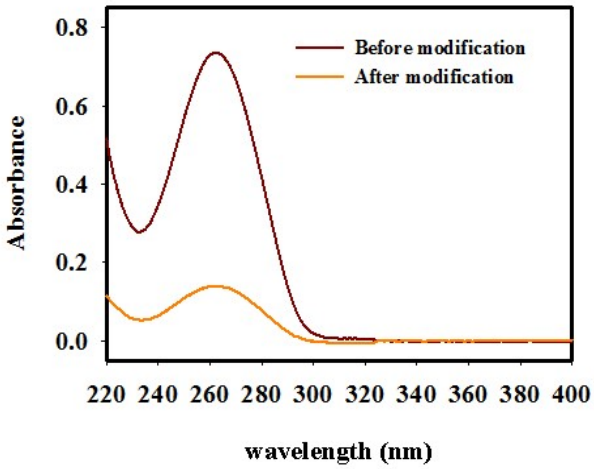

Figure S1. UV-vis absorbance of the CPs in the supernatant solution before and after modification on the surface of MMPs via streptavidin-biotin affinity.

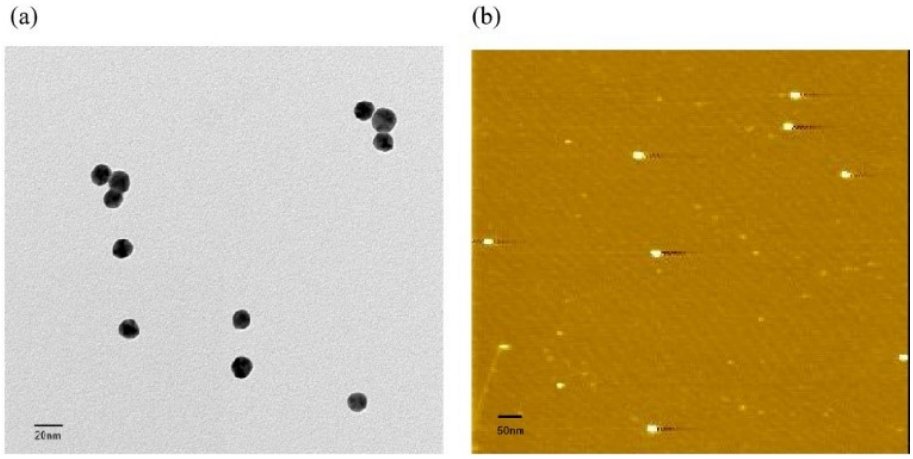

Figure. S2 TEM image (a) and AFM image (b) of 13 nm AuNPs synthesized by sodium citrate reduction method.

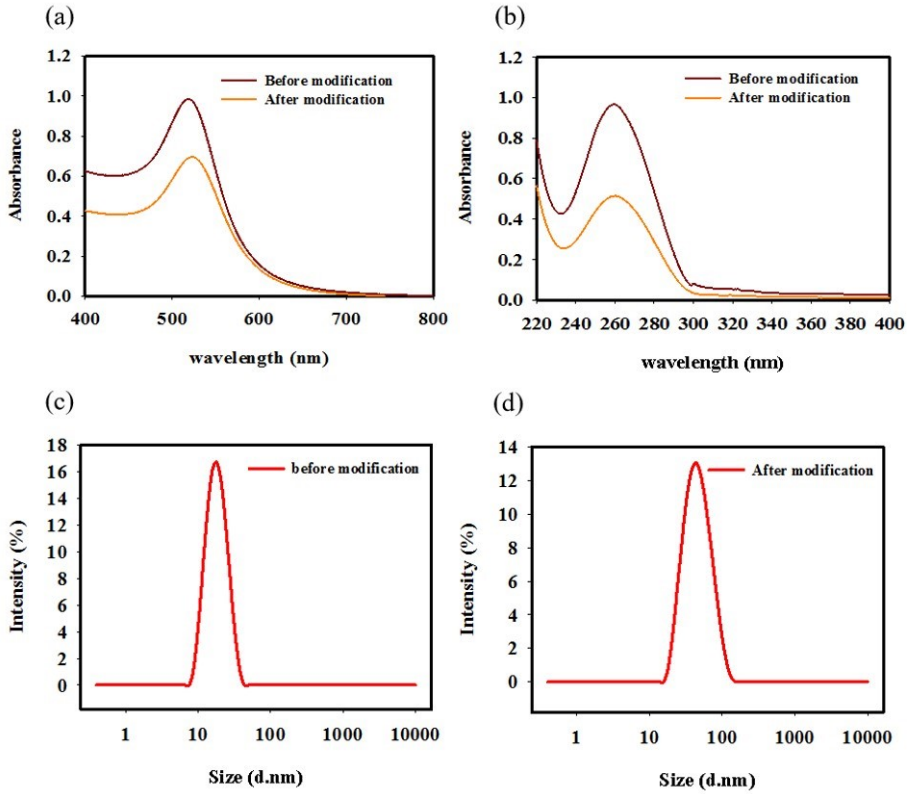

Figure S3. (a) UV-vis absorption spectra before and after AuNPs modification; (b) UV absorption spectra of thiolated DNA in the supernatant before and after modification; (c) The hydrated diameter size before AuNPs modification; (d) The hydrated diameter size after AuNPs modification.

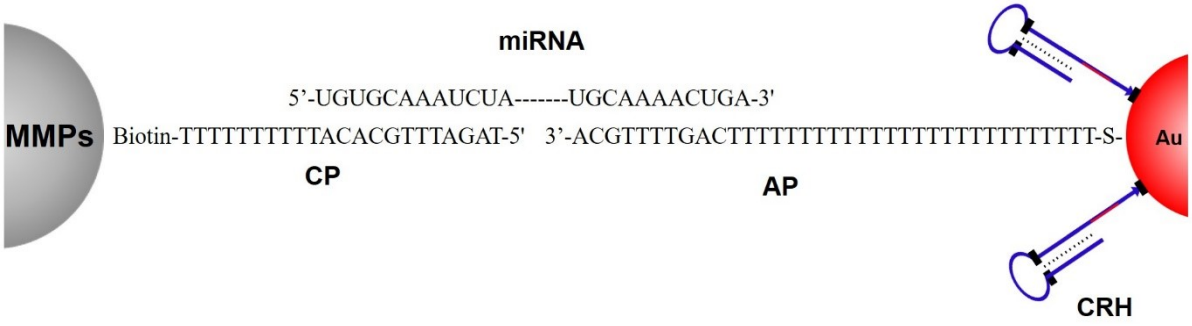

Figure S4. The sequence optimization of the sandwich hybridization between the CPs and APs.

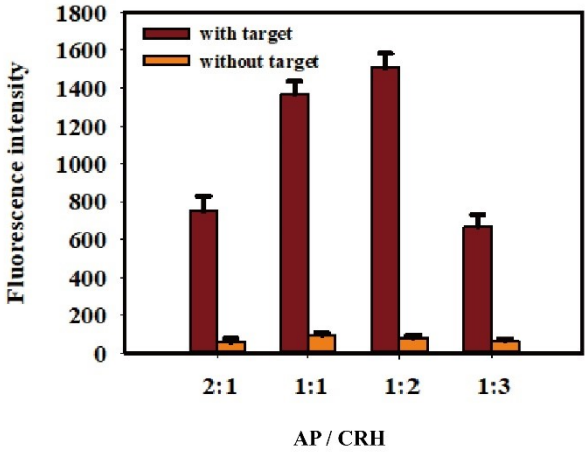

Figure S5. The optimization of the molar ratio of APs / CRHs. Four kinds of molar ratio including 2:1, 1:1, 1:2 and 1:3, were studied respectively under the same reaction time of 80 min. The fluorescence spectra were scanned with excitation wavelength of 488 nm and slit width of 5 nm, and all experiments were repeated three times in parallel.

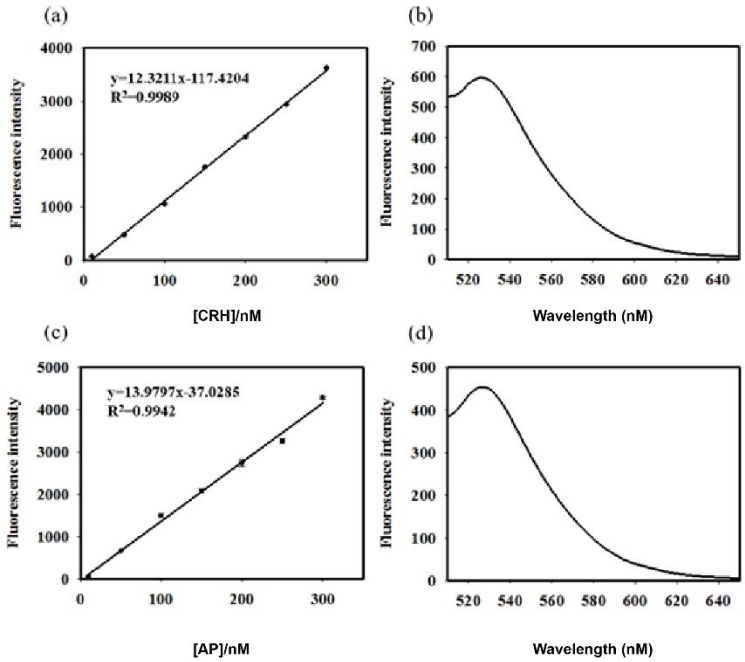

Figure S6. (a) The standard curve of fluorescence intensity as function of different concentrations of CRHs (10 nM, 50 nM, 100 nM, 150 nM, 200 nM, 250 nM, 300 nM); (b) Fluorescence spectra for CRHs quantification with excitation wavelength of 488 nm and slit width of 5 nm; (c) The standard curve of fluorescence intensity as function of different concentrations of APs (10 nM, 50 nM, 100 nM, 150 nM, 200 nM, 250 nM, 300 nM); (d) Fluorescence spectra of APs quantification with excitation wavelength of 488 nm and slit width of 5 nm.

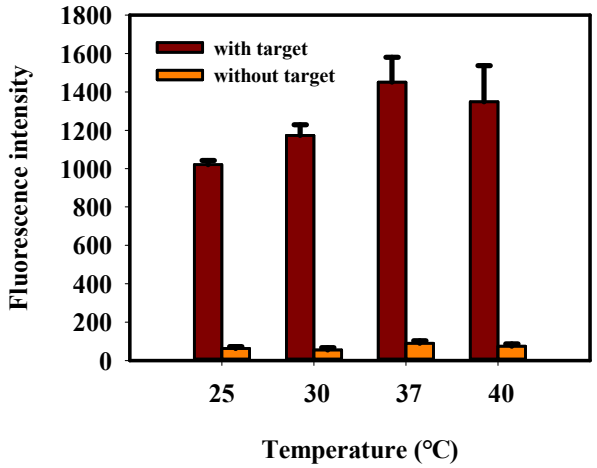

Figure S7. The optimization of incubation temperature in BCA amplification.

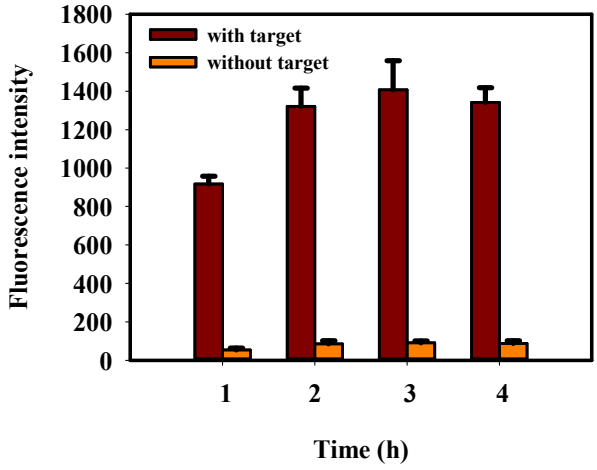

Figure S8. The optimization of enrichment time by MMPs in BCA amplification.

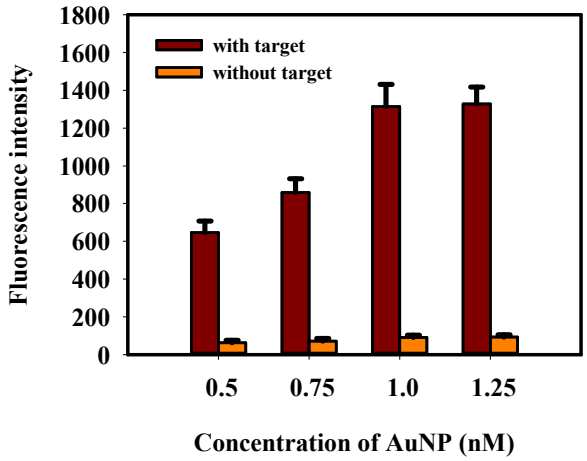

Figure S9. The optimization of the concentrations of AuNPs used in BCA amplification.

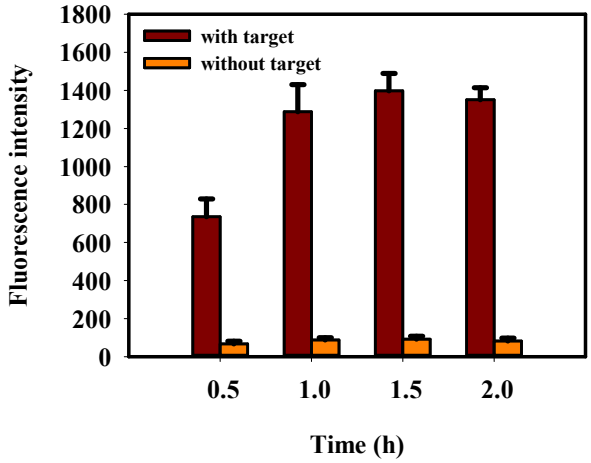

Figure S10. The optimization of the reaction time of forming sandwich structures after adding AuNPs.

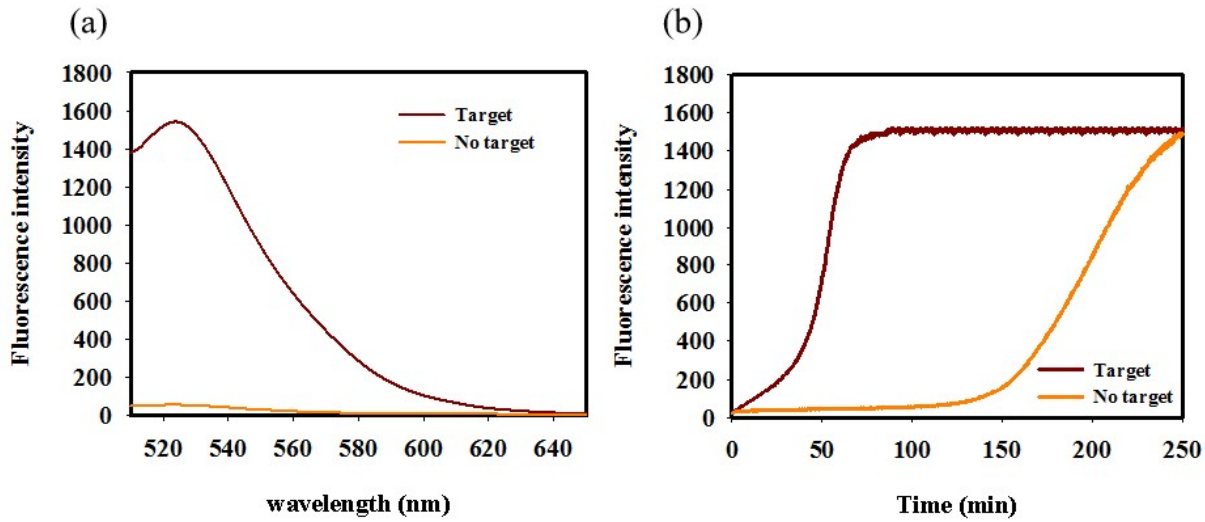

Figure S11. The feasibility study by fluorescence experiments. (a) Fluorescence spectra in the presence and absence of the target with a concentration of 5 nM, at the time point of 100 min; (b) Fluorescence time scanning in the presence and absence of the target with a concentration of 5 nM. Fluorescence excitation wavelength was set at 488 nm, with the slit width of 5 nm.

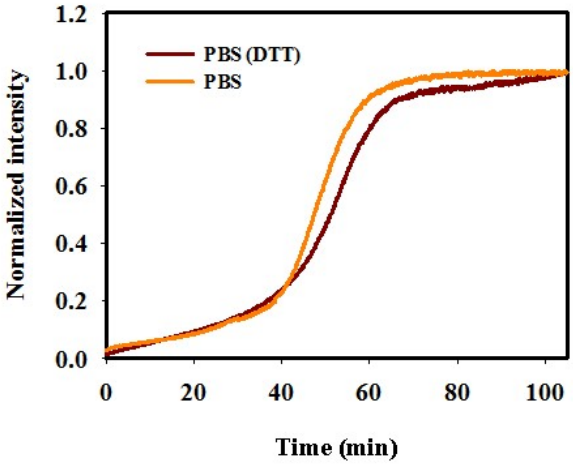

Figure S12. The investigation of the reaction buffer with or without DTT agent. Fluorescence time scanning were performed in PBS solution and PBS solution containing 20 mM DTT, respectively.

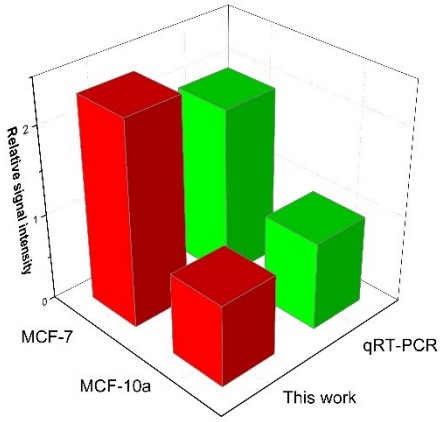

Figure S13. Comparison of miRNA-19a expression levels detected in cell lysates media using this method (red histogram) and qRT-PCR (green histogram). Relative intensity in MCF-7 cells was normalized by the results of qRT-PCR experiments in MCF-10a cells, of which the intensity was set at 1.

References

1. L. Lan, J. Huang, M. Liu, Y. Yin, C. Wei, Q. Cai and X. Meng, *Chem. Sci.*, 2021, **12**, 4509-4518.
2. J. Zhang, M. He, C. Nie, M. He, Q. Pan, C. Liu, Y. Hu, T. Chen and X. Chu. *Chem. Sci.*, 2020, **11**, 7092-7101.