Electronic Supplementary Information (ESI)

Auto-Cycling Primer Extension for Amplified MicroRNA Detection

Huimin Wang, Jiaoli Wang, Ruiting Liu, Zhenxiang Liu, Kemin Wang, Jin Huang*

State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Key Laboratory for Bio-Nanotechnology and Molecular Engineering of Hunan Province, Hunan University, Changsha, China.

*E-mail: jinhuang@hnu.edu.cn

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).

Polyacrylamide gel electrophoresis

Firstly, all hybrid complex and hairpins required were heated to 95℃ for 5 min, respectively, and then cooled down to room temperature for the following use. The CRH/blocker/miR-19a triple mixtures were treated at 37℃ for 2 h in 10 mM PBS (137 mM NaCl, 10 mM phosphate, 2.72 mM KCl, pH 7.4). The CRH and primer were incubated with 0.25 U/μL Bst DNA Polymerase, 2 mM dNTP, 2 mM $MgSO_4$, Bst reaction buffer in 10 mM PBS at 37℃ for 4 h and then heated at 80℃ for 20 min to make the enzyme inactive. The same method as above was used for the total reaction. Secondly, 12% polyacrylamide gel was prepared for running. 8 μL above samples were mixed with 2 μL 6×loading buffer and 2 μL 100× SYBR Gold. Finally, the samples were run at 120 V for 2 h in 1× TBE on ice and the result was analyzed by Azure C600 gel imaging system.

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.

Optimization of reaction conditions

In the auto-cycling primer extension reaction, the experiments consisted of the test experiment with DNA-19a and the control experiment without DNA-19a. First, the ratio of CRH probe to blocker probe was optimized. The concentration ratios of CRH to blocker were respectively 10/12 nM, 10/15 nM, 10 /18 nM and 10/21 nM. After incubating for 70 min on a thermostatic metal shaker bath at 37℃ and then heated at 80℃ for 20 min, the fluorescence spectra were measured. And other conditions of the experiments were the same as the above. Second, the concentration of Bst DNA Polymerase was optimized. The concentrations of Bst DNA Polymerase were respectively 0.025 U/μL, 0.05 U/μL, 0.125 U/μL, 0.25 U/μL, 0.5 U/μL. The reaction solution was transferred to a colorimetric dish, and the temperature was kept at 37℃ for fluorescence time scanning. The scanning time range was set to 0-6000 s. Finally, the concentration of Mg^{2+} and the reaction temperature were optimized, and the concentration of Mg^{2+} was changed to 1 mM, 2 mM, 3 mM and 4 mM respectively. Next, the reaction was carried out simultaneously at 25℃, 37℃, 50℃ and 65℃, respectively. After incubating for 70 min on a thermostatic metal shaker bath at 37℃ and then heated at 80℃ for 20 min, the fluorescence spectra were measured.

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, 200 pM, 50 pM, 20 pM, 5 pM, 0) 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.

Calculation of relative detection

In the specificity assay, the POI value of six miRNAs can be obtained from the time scanning curves. With the exception of miR-19b, the other four miRNAs barely respond, so their POI values can be derived from the background curve in sensitivity assay. Assuming that the detection efficiency of miR-19a(perfect matching)is 100%, the relative detection efficiency of each miRNA can be calculated based on the POI differences between the target and the other five miRNAs[1]. For example, the POI

values of miR-19a and miR-19b are 56 min and 79 min respectively, on account of the relative detection efficiency of miR-19a is 100, so the relative detection efficiency of miR-19b is 4.35.

MiR-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.

Supporting Tables:

Table S1. Oligonucleotide sequences used in this work.

Name	Sequence（5’-3’）
CRH	TGCGCATTTTTGCGCA**mGmG**GCCTTTTGGC**mCmC**TGCGCA AAAATGCGCAGTAGTGTAATGTGCAAATC-Inverted T
blocker	TCAGTTTTGCATAGATTTGCACATTACACTAC-Inverted T
DNA-19a	TGTGCAAATCTATGCAAAACTGA
DNA-19b	TGTGCAAATCCATGCAAAACTGA
DNA-17	ACTGCTGAGCTAGCACTTCCCGA
DNA-18a	ACTGCCCTAAGTGCTCCTTCTGG
DNA-20a	ACTGCATTATGAGCACTTAAAG
DNA-92a	TATTGCACTTGTCCCGGCCTGT
primer	CTTGGTTTACGTGTTTTACACTAC
Long-stem hairpin	CTTGGTTTACGTGTTTTACACTACTGCGCATTTTTGCGCAGT AGTGTAAAACACGTAAACCAAG
FP1-1	FAM-CTTGGTTTACGTGTTTTACACTAC
BQ1	AACACGTAAACCAAG-BHQ1
miR-19a	UGUGCAAAUCUAUGCAAAACUGA

mG, **mC** stand for 2’ -O-methyl G and 2’ -O-methyl C[2].

Supporting Figures:

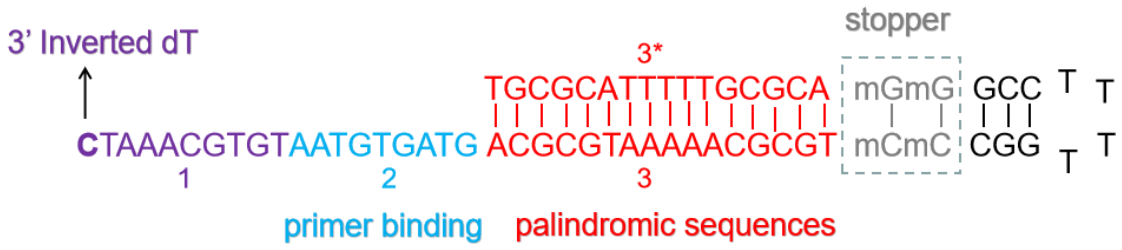

Figure S1. The design of CRH. Stopper (mG, mC) stand for 2'-O-methyl G and 2'-O-methyl C. Red sequences (part 3 and 3*) stand for palindromic sequences. Blue sequences (part 2) stand for primer binding region. The 3' of CRH is modified by inverted dT to avoid its extension.

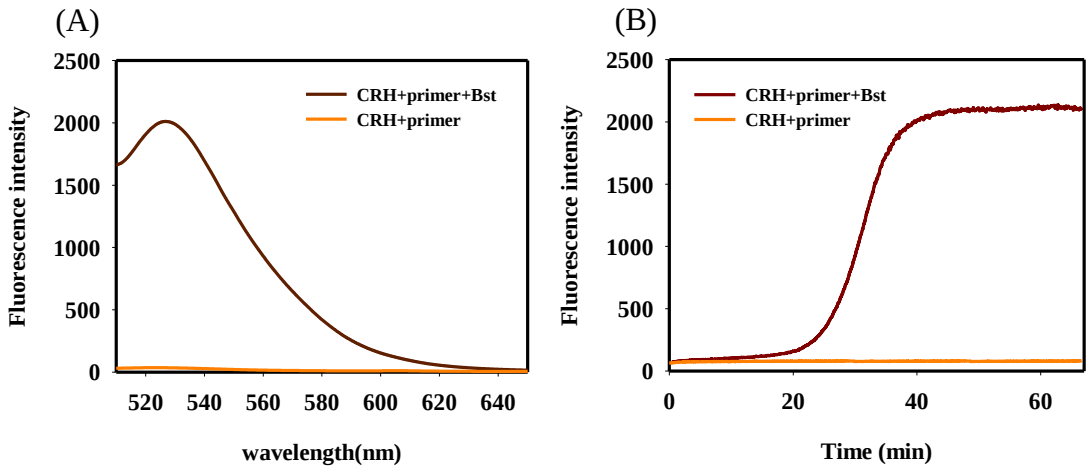

Figure S2. （A）Fluorescence wavelength scanning curves of primer, CRH+primer+Bst, and CRH+primer.（B）Real time fluorescence scanning curves of CRH+primer+Bst and CRH+primer.

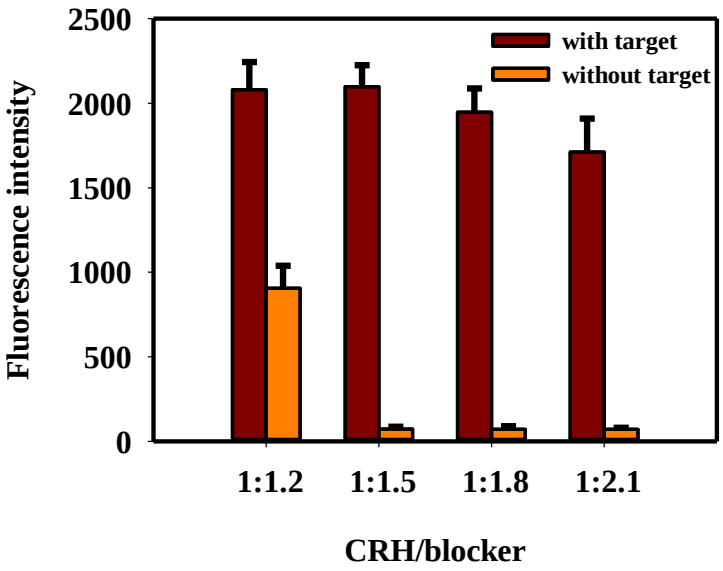

Figure S3. The optimization of CRH-to-blocker ratio. After incubating for 70 min on a thermostatic metal shaker bath at 37℃ and then heated at 80℃ for 20 min, the fluorescence spectra were measured.

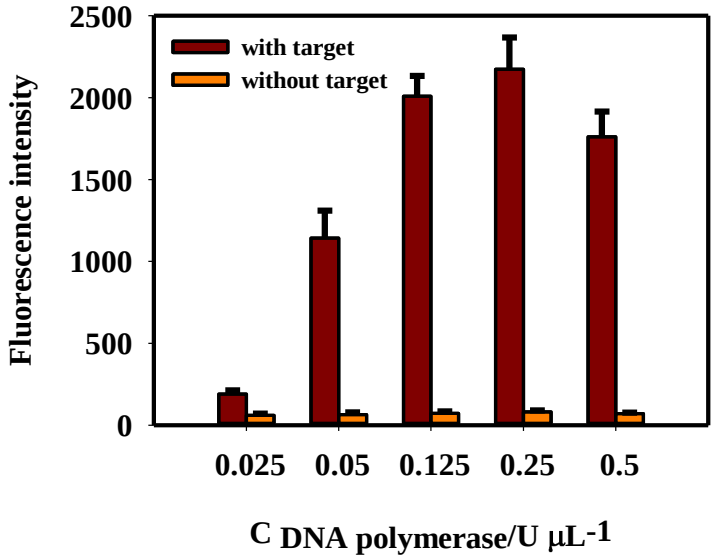

Figure S4. The optimization of the DNA polymerase concentrations. After incubating for 70 min on a thermostatic metal shaker bath at 37℃ and then heated at 80℃ for 20 min, the fluorescence spectra were measured.

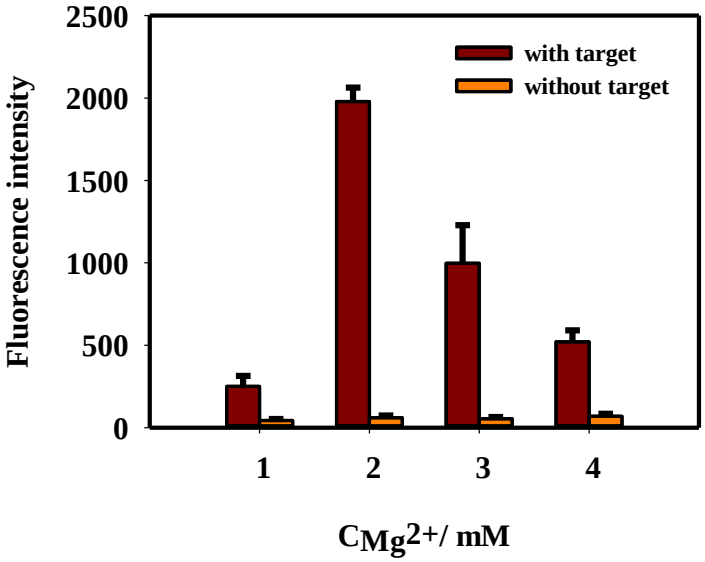

Figure S5. The optimization of magnesium ion concentration. After incubating for 70 min on a thermostatic metal shaker bath at 37℃ and then heated at 80℃ for 20 min, the fluorescence spectra were measured.

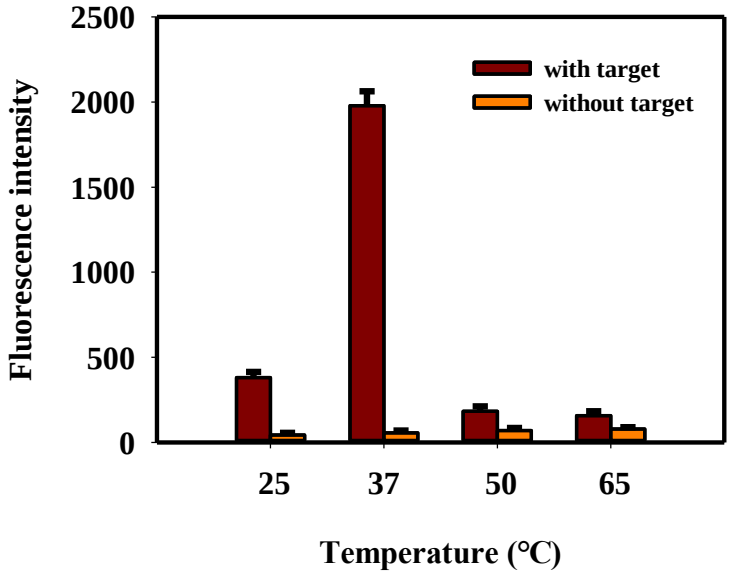

Figure S6. The optimization of reaction temperature. After incubating for 70 min on a thermostatic metal shaker bath at 37℃ and then heated at 80℃ for 20 min, the fluorescence spectra were measured.

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.