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Supporting Information

A universal rolling circle amplification for label-free and high-specific nucleic acids sensing

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S1. Reagents and materials

All oligonucleotides (**Table S1**) used in this study were synthesized by Sangon Biotech. Co., Ltd. (Shanghai, China) with HPLC purification. Thioflavin T (ThT), DEPC-treated water, deoxynucleotide triphosphates solution mixture (dNTP), 6 × loading buffer, 0.5 × TBE buffer and Tris-HCl buffer were also purchased from Sangon Biotech. Co., Ltd. (Shanghai, China). Phi 29 DNA polymerase, T4 DNA ligase, Exonuclease I, Exonuclease III and their corresponding buffers were all purchased from New England Biolabs (NEB, Beijing, China). Other analytical grade reagents such as KCl and $MgCl_2$ were purchased from Sigma-Aldrich Chemical Co., Ltd (St. Louis, USA) and used without further purification. All the solutions and deionized water used in this study were treated with DEPC and autoclaved to avoid enzymatic hydrolysis of nucleic acids.

Table S1 Sequences of the used oligonucleotides

Name	Sequence(5'- 3')
Ligation probe	GAT GTT GAA GTA GGA TGT CCG C
Padlock probe	5'P-ACT TCA ACA TCA AAC CCA CCC GCC CTA CCC ACA AAT CTA ATC ATA TAA GCG GAC ATC CT
miR-221	AGC TAC ATT GTC TGC TGG GTT TC
Blocker	GAA ACC CAG CAG ACA ATG TAG CTC G
HP	5'P-AAG TAG GAT TAC GAG CTA CAT TGT CTG CTG CCG CTC GTA GTC
miR-222	AGC TAC ATC TGG CTA CTG GGT CTC
Blocker I	GAG ACC CAG TAG CCA GAT GTA GCT GC
HP I	5'P-AAG TAG GAT TAG CAG CTA CAT CTG GCT ACT GAC GCT GCT AGT C
mis_1-221	AGC TAC ATT CTC TGC TGG GTT TC
mis_2-221	AGC TAC ATT GTC TGC TGG CTT TC
mis_3-221	AGC TAC ATT CTC TGC TCG GTT TC
mis_1-222	AGC TAC ATC TGG GTA CTG GGT CTC
mis_2-222	AGC TCC ATC TGG CTA CTG GGT CTC
mis_3-222	AGC TAC ATC TGG CTA CTG GCT CTC
miR-155	TTA ATG CTA ATT GTG ATA GGG GT
LP	5'P-AAG TAG GAT GTC

S2. Preparation of the CDT and BHP

20 μL of 10 μM ligation probe and 20 μL of 10 μM padlock probe were heated to 95 ℃ for 5 min and then gradually cooled down to 25 ℃. Afterwards, a volume of 50 μL mixture consisted of 1000 U T4 DNA ligase, 1 × T4 buffer and DEPC-treated water was added and incubated at 16 ℃ for 6 h, followed by heating at 65 ℃ for 10 min to terminate the ligation reaction. Next, 20 U Exonuclease I and 150 U Exonuclease III were added and kept at 37 ℃ for 17 h to digest extra ligation probes and the un-ligated template. Finally, the resulting 100 μL mixture was heated at 80 °C for 20 min to stop the digestion reaction and the CDT was synthesized successfully (**Fig. S1**).

For the synthesis of BHP, the probes of blocker and HP were mixed at the molar ratio of 1:1, heated in a water bath at 95 ℃ for 5 min, and then cooled slowly to room temperature to obtain the double-stranded blocker-hairpin primer (BHP) probe (**Fig. S2**).

The obtained CDT and BHP were diluted with DEPC-treated water to 500 nM and 200 nM respectively and stored at -20 ℃ for further use.

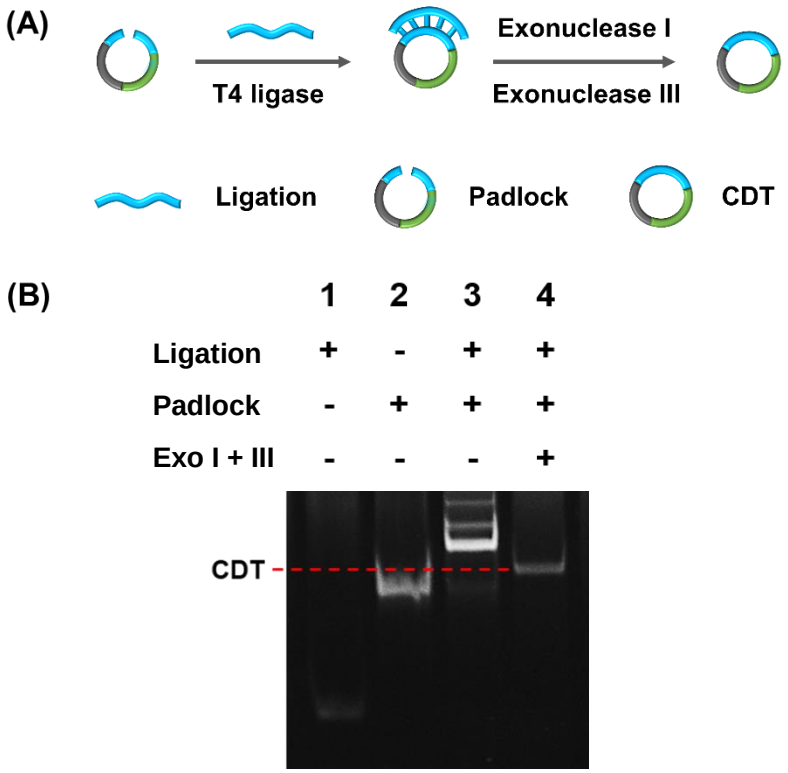

Fig. S1. (A) Principle and (B) Polyacrylamide gel electrophoresis verification of CDT synthesis.

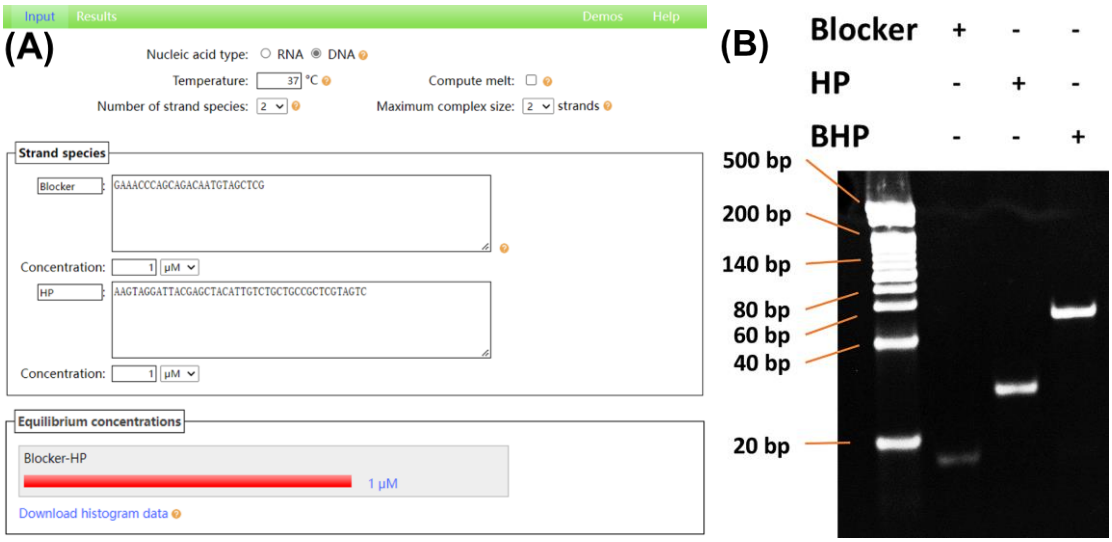

Fig. S2. (A) The theoretical hybridization efficiency of blocker and HP. (B) Polyacrylamide gel electrophoresis verification of BHP synthesis.

S3. Polyacrylamide gel electrophoresis (PAGE) analysis

Different DNA solutions (10 μL per well) mixed with 2 μL 6 × loading buffer, which were injected into the lanes of a 10% polyacrylamide gel in 0.5 × tris-borate-EDTA (TBE) buffer. The PAGE analysis was performed at a constant voltage of 250 mV for 30 min, after which the gel was stained with 4S GelRed and visualized via DigiGenius gel system (Syngene, UK).

S4. Fluorescent detection of miRNA

The 25 μL reaction system including various concentrations of miRNA, 50 nM CDT, 0.1 mM dNTP, 10 nM BHP, 2.5 U of phi 29 polymerase, 1× phi 29 reaction buffer, was incubated at 37 ℃ for 2 hours and then heated to 80 ℃ for 20 min to terminate the amplification reaction. Afterwards, 240 μM ThT, 1.5 M KCl and DEPC-treated water were added to the above reaction solution to make up the total volume to 50 μL, and kept at 37 °C for 30 min. Then the emission spectra were obtained in the range from 465 to 600 nm with an excitation wavelength of 450 nm by using a fluorescence spectrophotometer (WATERS Prep 150, Waters Corporation, USA). The fluorescence intensity was recorded at the emission wavelength of 488 nm to evaluate the performance of the proposed URCA system. Both the excitation and emission slit widths were set as 2.5 nm.

S5. Calculation of the LOD for miRNA sensing

The detection limit can be used to estimate the minimum target concentration that can be detected by the developed method, which can be calculated by the following formula:

$$\mathrm{Lg}c_{LOD} = 3\sigma/k$$

Herein, k means the slope of the regression equation. σ represents the standard deviation of the multiple measured signals of the blank, n=11.

S6. Exploration of the effect of HP on the progress of phi29 DNA polymerase

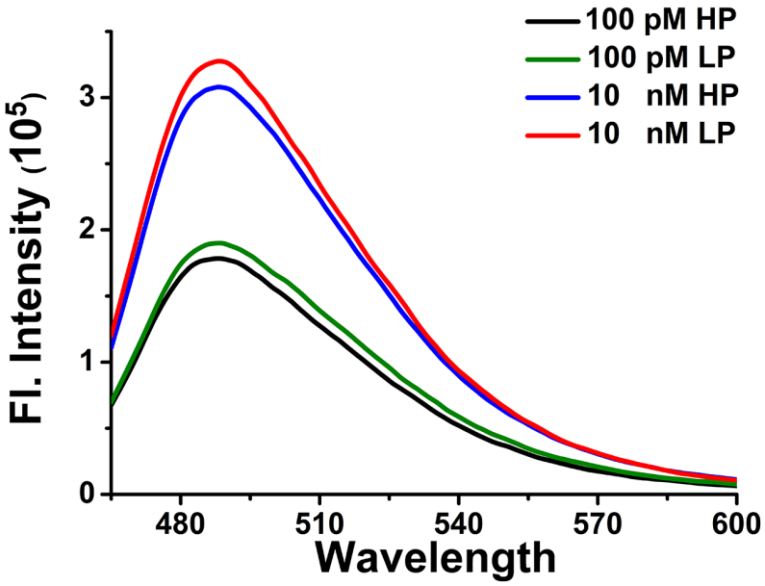

Fig. S3. The fluorescence intensity after incubation of the same concentration of LP and HP with CDT respectively.

S7. Optimization of experiment conditions

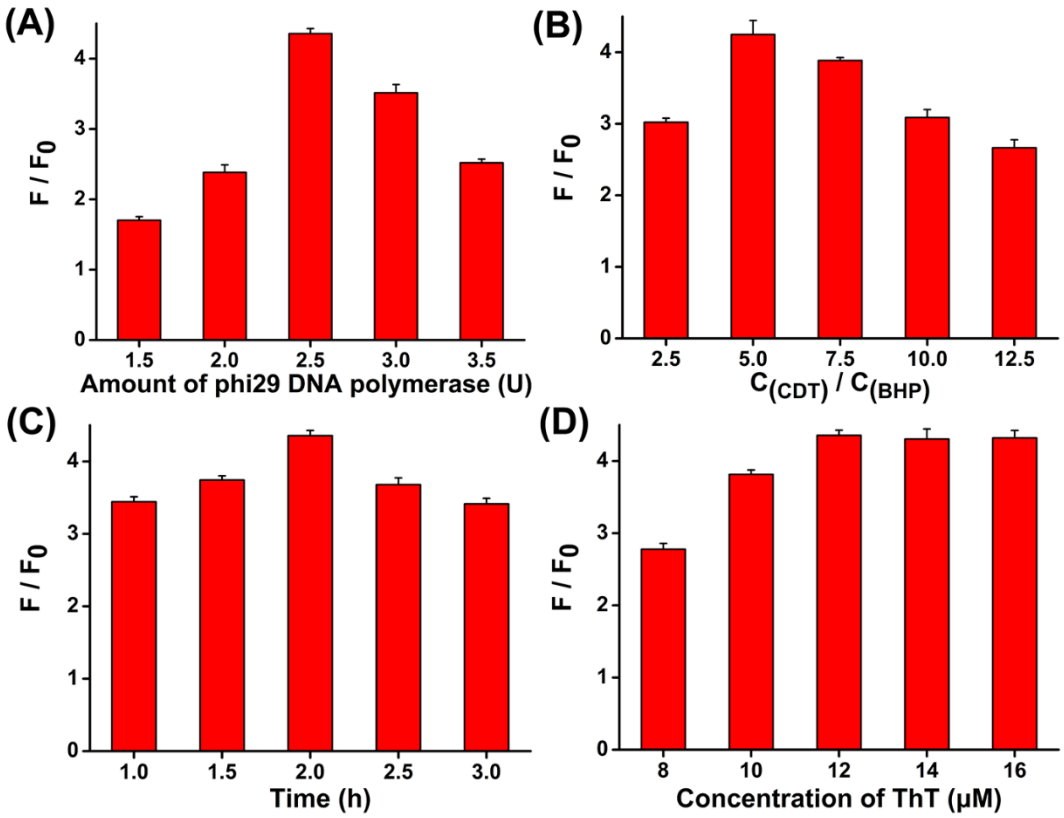

Fig. S4. Optimization of the assay parameters with (A) phi29 DNA polymerase amount, (B) Concentration ratio of CDT to BHP, (C) RCA reaction time and (D) ThT concentration. F represents the fluorescence intensity with target, and F_0 is the blank fluorescence intensity without target. (n = 3).

S8. Verification of the feasibility of universal detection

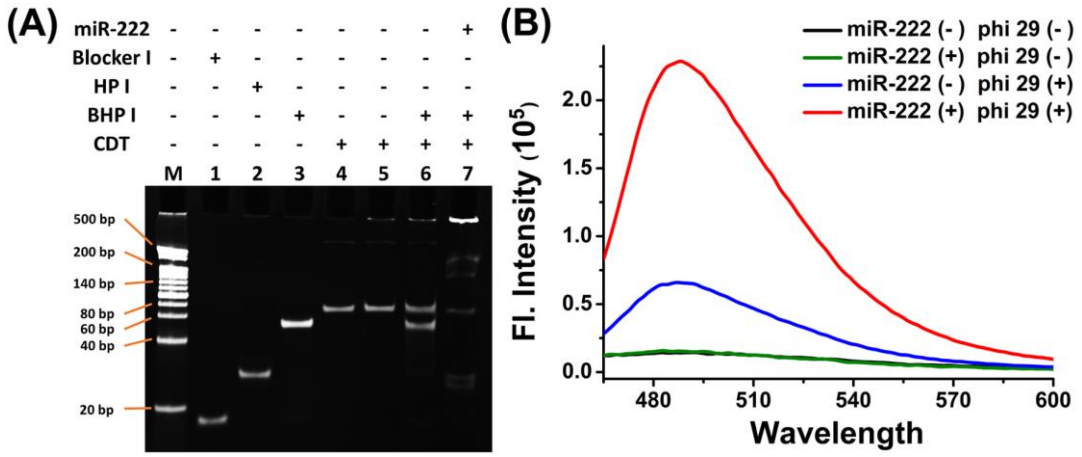

Fig. S5. (A) Polyacrylamide gel electrophoresis analysis of different samples (lane 5, 6, 7 with dNTP and phi 29 added). (B) Fluorescence spectra under different conditions (all with CDT, BHP I and dNTP added).

S9. Evaluation of the specificity of the URCA for miR-222 sensing

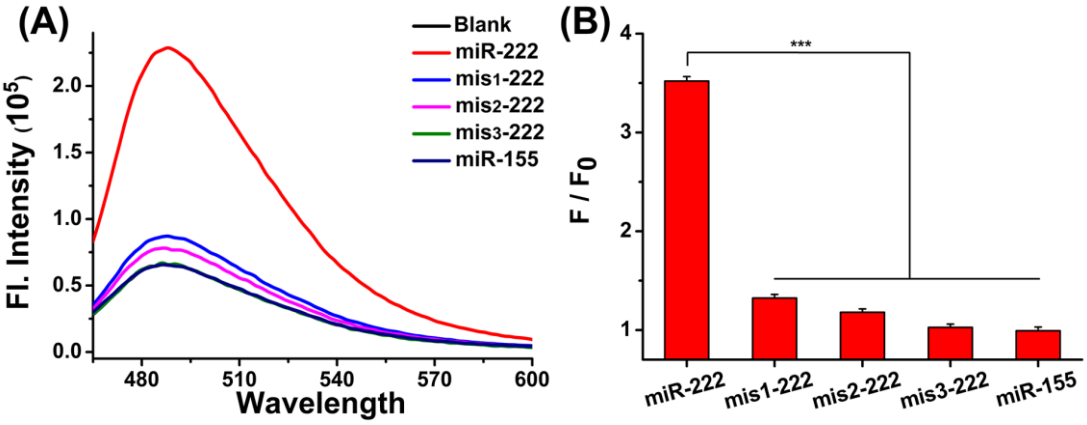

Fig. S6. (A) Fluorescence spectra produced by miR-222 and other interference nucleic acids (mis_1-222, mis_2-222, mis_3-222, miR-155, all with 50 nM concentrations). (B) F/F_0 of different nucleic acids present. F is the fluorescence intensity when the target is present, and F_0 is the blank fluorescence intensity when the target is absent. (*** means $p < 0.001$, $n = 3$).

S10. Comparison with the previously reported RCA-based methods for nucleic acid detection

Table S2 Comparison with the previously reported RCA-based methods for nucleic acid detection

Strategy	Output Signal model	LOD (pM)	Reference
Self-primed nicking endonuclease assisted RCA	Electrochemistry	0.1	1
Hairpin-mediated padlock cyclization RCA	Labeled fluorescence	0.1	2
Increasingly branched RCA	Labeled fluorescence	0.1	3
Ligation-Free RCA	Labeled fluorescence	0.48	4
Toehold-initiated constructure-switchable RCA	Labeled fluorescence	0.89	5
This work	Label-free fluorescence	1.25	This work
		1.35	

References

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