Supporting Information Available

Homogeneous and Sensitive Detection of microRNA with Ligase Chain Reaction and Lambda Exonuclease-assisted Cationic Conjugated Polymer Biosensing

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Table S1. Sequences of Probes and Target miRNAs (5′-3′) Used in the Experiments*

let-7a	UGAGGUAGUAGGUUGUAUAGUU
let-7c	UGAGGUAGUAGGUUGUAU**G**GUU
let-7f	UGAGGUAGUAG**A**UUGUAUAGUU
miR221	AGCUACAUUGUCUGCUGGGUU
Probe X	TAATACGTTACCTCAAAAGAAAACTATACAAC
Probe X′	TAATACGTTACCTCAAAAGAAAACTATACArArC
Probe Xr	P-GTTGTATAGTTTTCTTTTGAGGTAACGTATTA-FAM
Probe Y	P-CTACTACCTCACTGACAGAAAAGCAAACA-FAM
Probe Yr	TGTTTGCTTTTCTGTCAGTGAGGTAGTAG

*The two 3′-terminals (underline rArC) bases of probe X′ are ribonucleotides. The 5′-terminals P in probe Xr and probe Y are 5′-phosphorylated modification. The underline and bold bases in let-7c and let-7f are the mismatched bases with let-7a. 3′-terminals FAM in probe Xr and probe Y are fluorescein-labeled bases.

Materials. T4 RNA ligase 2 was purchased from New England Biolabs (USA). Thermostable Ampligase was purchased from Epicentre Technologies (Madison, WI). Lambda exonuclease and RiboLock RNase Inhibitor were purchased from Thermo Sciencitic (USA). The human lung total RNA sample and ribosomal RNA from *E. coli* were purchased from Life Technologies (USA). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was obtained from Sigma. The cationic poly[(9,9-bis(6-N,N,N-trimethylammonium)hexyl) fluorenylphenylene dibromide] (PFP) used as the CCP in the FRET experiments was perpared according to the procedure described in the literature.[1] The chemical structure of CCP was shown in Figure S1. The synthetic oligonucleotides and miRNAs, diethypyrocarbonate (DEPC)-treated deionized water, used in this study were obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The sequences of the oligonucleotides and miRNAs were listed in Table S1. All the oligonucleotides and miRNAs were purified by HPLC. PCR tubes and pipet tips used in miRNA assay were purchased from Axygen (USA). All solutions were prepared in DEPC-treated deionized water. The other reagents were of analytical reagent grade and used as purchased without further purification.

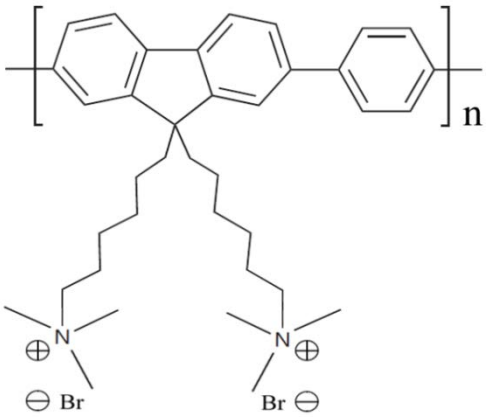

Figure S1. Chemical structure of CCP

Instruments. A 2720 thermal cycler (Applied Biosystems) was used to perform the LCR reaction. A Hitachi F-4500 spectrofluorometer (Tokyo, Japan) equipped with a xenon lamp was used to obtain the fluorescence spectra.

Ligation reaction with T4 RNA ligase 2. The ligation reaction with T4 RNA ligase 2 was performed with a 10 μL reaction mixture containing 1× ligation buffer of T4 RNA ligase 2 (50 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 400 μM ATP (pH 7.8)), 40 U RiboLock RNase Inhibitor, 1 nM probe X and 1 nM probe Y, and an appropriate amount of the target miRNA. Before adding T4 RNA ligase 2, the reaction mixture was denatured at 95 °C for 3 min and cooled slowly to 39 °C over a 20 min period. After annealing, 1 U T4 RNA ligase 2 was added to the mixture and incubated at 39 °C for 1 h. Moreover, to prevent the degradation of RNA, the labware consumables used in miRNA assays including PCR tubes and pipet tips should be sterile, DNase-free, and RNase-free.

LCR reaction. The LCR reaction was carried out in 20 μL mixture containing 10 μL ligation product of T4 RNA ligase 2, 20 mM Tris-HCl (pH 8.3), 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD, and 0.01% Triton® X-100, 50 nM probe X, Y, Xr, and Yr, respectively. After denatured at 95 ºC for 3 min, 1 U ampligase was rapidly added to the reaction mixture at 75 ºC. The LCR reaction was achieved with following 30 thermal cycles at 95 °C for 30 s and 65 °C for 30 s.

Lambda Exonuclease digestion and fluorescence measurement. In 20 μL LCR product mixture, 10 U lambda exonuclease was added to digest the unreacted 5′-phosphorylated probe Y and Xr by incubation at 37 °C for 90 min. The digestion reaction was terminated by incubation at 85 °C for 15 min and subsequently held at 4 °C. Afterwards, aliquots of 4 μL of LCR product and 2 μL of CCP (15 μM in monomer repeat units, RUs) were then added to a 1.5 mL centrifuge tube and diluted to 200 μL with 25 mM HEPES buffer (pH 8.0). The fluorescence spectra were

measured in a quartz micro cell. The excitation wavelength was 380 nm, and the spectra were recorded between 400 and 600 nm.

Optimization Experimental Conditions for miRNA Detection.

To explore the potential of the proposed assay for detection of target miRNA, the experimental conditions of LCR including the concentration of DNA ligase, the temperature, and cycle number of ligation reaction were studied and optimized.

Table S2. Effect of content of ampligase on FRET efficiencies in LCR for let-7a detection*

Concentration of ampligase (U/μL)	0.025	0.05	0.1	0.2
FRET efficiencies (%) of blank	33.3	37.79	45.32	55.86
FRET efficiencies (%) of let-7a	35.79	67.73	69.72	85.16
Relative FRET efficiencies (%)	2.49	29.94	24.4	29.3

* The final concentration of let-7a in fluorescence measurement was 10 fM. The blanks were treated without let-7a template and detected in the same way as the let-7a.

Ampligase, one thermostable DNA ligase, was chosen in LCR with high specificity and efficiency. As shown in Table S2, the FRET efficiency produced by the let-7a was increased significantly with an increase of the concentration of ampligase in LCR from 0.025 U/μL to 0.05 U/μL and increased little by little from 0.05 U/μL to 0.2 U/μL. However, the FRET efficiencies produced by the blank without let-7a kept solwly increasing with the concentration of ampligase from 0.025 U/μL to 0.2 U/μL. The relative FRET efficiencies, in which the blank value was subtracted, reached the maximum value with the concentration of ampligase at 0.05 U/μL, which was used in subsequent experiments.

Table S3. Effect of reaction temperature on FRET efficiencies in LCR for let-7a detection*

Reaction temperature (T/°C)	55	60	65	70
FRET efficiencies (%) of blank	52.56	25.51	25.52	19.88
FRET efficiencies (%) of let-7a	61.89	52.99	51.06	26.4
Relative FRET efficiencies (%)	9.33	27.48	25.54	6.52

* The final concentration of let-7a in fluorescence measurement was 10 fM. The blanks were treated without let-7a template and detected in the same way as the let-7a.

The different temperatures of the ligation reaction in LCR have a crucial effect on the FRET efficiency for miRNA detection. Table S3 showed that the FRET efficiencies from both let-7a and blank reached relatively high value with the ligation temperature at 55 °C, which suggested the poor selectivity for let-7a detection. The FRET efficiencies for let-7a reduced gradually with the increase of the ligation temperature from 55 to 65 °C and reduced remarkably from 65 to 70 °C in LCR. By contrast, the FRET efficiencies for blank reduced noticeably with the increase of the ligation temperature from 55 to 60 °C and reduced slowly from 60 to 70 °C. The maximum value of the relative FRET efficiency was obtained with the ligation temperature at 65 °C. In consideration of the specificity and sensitivity for miRNA detection, the ligation temperature at 65 °C in LCR was selected throughout subsequent experiments.

The sensitivity for miRNA detection depends on the thermal cycle number of LCR. Table S4 showed that the FRET efficiency from miRNA was increased remarkably with increasing the thermal cycle number of LCR from 20 to 40. However, the FRET efficiency of blank was increased little with the thermal cycle number of LCR from 20 to 30, which suggested the execllent specificity for detecion of miRNA. When the cycle number was greater than 30, the

FRET efficiency of the blank increased significantly leading to the poor specificity of LCR for detection of miRNA. Therefore, the thermal cycle number of the LCR was set to 30 in subsequent experiments.

Table S4. Effect of the thermal cycle number of LCR on FRET efficiencies for let-7a detection*

Cycle number of LCR	20	25	30	35	40
FRET efficiencies (%) of blank	16.6	17.52	25.05	53.67	69.42
FRET efficiencies (%) of let-7a	21.85	54.62	66.9	71.28	85.1
Relative FRET efficiencies (%)	5.25	37.1	41.85	17.61	15.68

* The final concentration of let-7a in fluorescence measurement was 10 fM.The blanks were treated without let-7a template and detected in the same way as the let-7a.

Reference

(1) Duan, X.; Yue, W.; Liu, L.; Li, Z.; Li, Y.; He, F.; Zhu, D.; Zhou, G.; Wang, S. Single-nucleotide polymorphism (SNP) genotyping using cationic conjugated polymers in homogeneous solution. *Nat. Protoc.* **2009**, *4*, 984-991.