Electronic Supplementary Information

Simple and sensitive detection of microRNAs with ligase chain reaction

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Experimental Section

Materials and Reagents

T4 DNA ligase, Taq DNA ligase, and T4 RNA ligase 2 were purchased from New England Biolabs. PAGE-purified DNA oligonucleotides were obtained form Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). HPLC-purified miRNAs and 20 bp DNA Ladder Marker were purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). Agarose M was purchased from Bio Basic INC. Gold View (fluorescent dye for gel imaging) was purchased from Shanghai SBS Genetech Co. Ltd. (Shanghai, China). All the solutions for ligation reactions were prepared in DEPC-treated deionized water. All other reagents were of analytical reagent grade and used as purchased without further purification.

Apparatus

Px 2 Thermal cycler (Thermo Electron Co., USA) was used to control the temperature of ligation reactions and to perform the thermal cycles for ligase chain reactions (LCR). DYY-10 Electrophoresis Instrument (Liu Yi Instrument Factory, Beijing, China) was used for running the gel electrophoresis. Gel images were acquired with a versa Doc^{TM} 4000 imaging system (Bio-Rad, USA). Imaging analysis and pixel intensity measurement were performed with Quantity One software running under Windows XP.

Ligation of DNA probe A and probe B templated by miRNA

For the ligation reaction with T4 RNA ligase 2, the reaction mixture consisted of ligation buffer (50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1mM DTT, 400 μM ATP), 5U T4 RNA ligase 2, 200 nM DNA probe A, 200 nM DNA probe B, and appropriate amount of target miRNA in a reaction volume of 10 μL. For the ligation reactions with T4 DNA ligase, the reaction mixture consisted of ligation buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 μM ATP). 5U or 200U T4 DNA ligase,

200 nM DNA probe A, 200 nM DNA probe B, and appropriate amount of target miRNA in a reaction volume of 10 μL.

To perform the ligation reaction, the DNA probe A, probe B, and target miRNA were firstly mixed. The mixture was heated at 80℃ for 2 min and at 37℃ for 5 min to perform the hybridization. Then the ligase buffer and ligase were added in the mixture, and the reaction mixture was incubated at 37℃ for 55 min to complete the ligation reaction. After the ligation reaction, the products were put on ice.

LCR amplification reaction

2 μL of the product of above ligation reaction was transferred to LCR reaction mixture with final volume of 20 μL containing the reaction buffer (20 mM Tris-HCl, pH 7.6, 10 mM $Mg(Ac)_2$, 25 mM KAc, 1 mM NAD, 0.1% Triton X-100), 200 nM DNA probe A, 200 nM DNA probe B, 200 nM DNA probe A', 200 nM DNA probe B'. The reaction mixture was heated at 94℃ for 1 min and 60℃ for 5 min. Taq DNA ligase was added in the reaction mixture at 60℃. The LCR reaction was carried out with following 20 thermal cycles : 94℃ for 1 min and 60℃ for 5 min.

Gel electrophoresis and imaging

5 μL of LCR reaction product was firstly mixed with 1 μL 6X Loading Buffer. The mixture was transferred into 4% agarose gel containing Gold View dye. The gel electrophoresis was performed at 100 V in TAE buffer (40 mM Tris-HCl, pH 8.5, 2 mM EDTA).

Optimization of the amount of T4 RNA ligase 2

20 pM let-7a miRNA was detected with the LCR assay by using different amount of T4 RNA ligase 2 for ligation of probe A and probe B templated by the miRNA. The blank was treated in the same way for LCR assay without let-7a. As shown in Fig. S1, as the amount of T4 RNA ligase 2 increase from 5 unit to 10 unit, the pixel intensity of the band produced by let-7a can not be obviously increased, indicating that 5 unit T4 RNA ligase 2 is enough for the ligation. Therefore, 5 unit T4 RNA ligase 2 is adopted for the LCR assay.

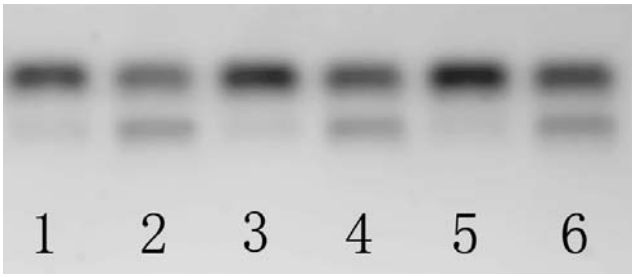


Fig. S1. *The influence of the amount of T4 RNA ligase 2 on the detection of let-7a with LCR assay. 20 pM let-7a and the blank without let-7a were detected according to the experimental procedure described in the experimental section except the amount of T4 RNA ligase 2. Lane 1, 3, 5: blank, lane 2, 4, 6: 20 pM let-7a. The amount of T4 RNA ligase 2: lane 1, 2: 5 U, lane 3, 4: 7 U, lane 5, 6: 10 U.*

Effect of the ligation temperature in the thermal cycles.

The DNA probe A and DNA probe B were firstly ligated at 37°C with 5 unit T4 RNA ligase 2 by using 20 pM let-7a as the template. Then the ligation product was amplified with LCR reaction at different ligation temperature from 55°C to 65°C in the thermal cycles. As shown in Fig. S2, the pixel intensity of the band produced by let-7a at the ligation temperature of 55°C, 60°C and 65°C are 671, 798, and 392, respectively. The ligation temperature of 65°C is near the *Tm* value of the DNA probes, which is estimated to be about 67°C, leading to the low ligation efficiency. Therefore, 60°C was used for the ligation in the thermal cycles of LCR reaction.

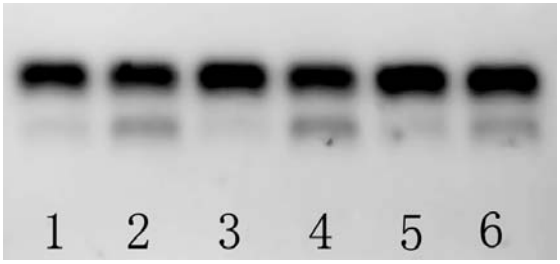
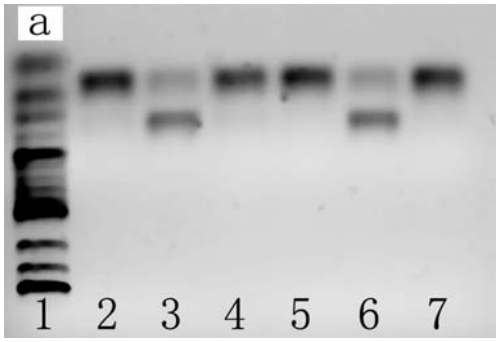


Fig. S2. *The effect of ligation temperature in the thermal cycles on the LCR assay. The detection of let-7a and the blank without let-7a was performed according to the procedure described in the experimental section except the ligation temperature in the thermal cycles. The ligation temperature is 55*°C *(lane 1, 2), 60*°C *(lane 3, 4), and 65*°C *(lane 5, 6), respectively. Lane 1, 3, 5: blank, lane 2, 4, 6: 20 pM let-7a.*

Validation of the universal practicability of the LCR-based assay.

To evaluate the universal practicability of the LCR assay for miRNA detection, mir-122 is randomly chosen because its sequence is unrelated to that of let-7a. According to the procedure described in experimental section, let-7a and mir-122 miRNA were detected with the LCR assay by using the specific DNA probes respectively corresponding to let-7a and mir-122. The sequences of mir-122 miRNA and the specific DNA probes (probe a, b, a′, b′) were shown in Fig. S3b. As demonstrated in Fig. S3a, the well-defined mir-122 band can be detected by using the mir-122-specific DNA probe a, b, a′, b′, while the band produced by let-7a can not observed. On the other situation, the well-defined let-7a band can be detected by using the let-7a-specific DNA probe A, B, A′, B′ (see the sequences in Fig. 1b), while the band produced by mir-122 can not

observed. The results indicate that the LCR-based assay is universally practical for detection of different miRNAs and has high specificity.



b
mir-122 5'-UggAgUgUgACAAUggUgUUUG-3'
a : 5'-TAATACgTTACCTCAAAAgAACAAACACCATT-3'
b : 5'-PO_4-gTCACACTCCACTgACAgAAAAAgCAAACATC-3'
a' : 5'-PO_4- AATggTgTTTgTTCTTTTgAggTAACgTATTA-3'
b' : 5'-gATgTTTgCTTTTTCTgTCAgTggAgTgTgAC-3'

Fig. S3. *(a) Detection of let-7a and mir-122 with the LCR-based assay by using the specific DNA probes respectively corresponding to let-7a and mir-122. Lane 1: DNA ladder marker. Lane 2, 3, 4: the detection results with LCR assay by using Let-7a-specific DNA probes, lane 2: blank, lane 3: 200 pM let-7a, lane 4: 200 pM mir-122. Lane 5, 6, 7: the detection results with LCR assay by using mir-122-specific DNA probes, lane 5: blank, lane 6: 200 pM mir-122, lane 7: 200 pM let-7a .(b) The sequences of mir-122 miRNA and the mir-122-specific DNA probes. The 11 bases of mir-122 at 3' (dotted) 5' (underlined) terminal are respectively complementary to the 11 bases at 3' terminal (dotted) of probe a and at 5' terminal (underlined) of probe b. The probe a' and probe b' are respectively complementary to probe a and probe b.*

Determination of let-7a in total RNA sample

Human lung total RNA (100μg/100μL, purchased from Applied Biosystem) was diluted to 100ng/μL with DEPC-treated water. Afterwards, aliquots of the diluted total RNA sample (2 μL) were respectively spiked with the initial ligaion reaction mixtures respectively containing standard synthetic let-7a at concentration of 0, 50, 100, and 200 pM with final volume of 10 μL. Next, the ligation reaction and the LCR amplification were performed step by step as described in the experimental section. The concentrations of the standard synthetic let-7a in the final LCR reaction mixtures are 0, 5, 10, and 20 pM, respectively. As shown in Fig. S4a, the let-7a can be clearly detected with the LCR assay and the results can be further verified by the addition of synthetic let-7a. As indicated in Fig. S4b, the amount of let-7a in final LCR reaction mixture can be estimated to be 10.8 pM by applying the standard addition method.

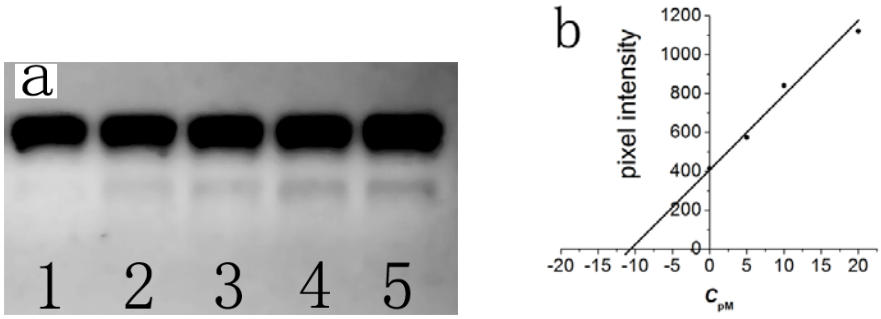


Fig. S4*. (a) Detection results of let-7a in total RNA sample. Lane 1: blank without total RNA sample and let-7a, lane 2: total RNA sample only, lane 3: total RNA sample + 5 pM let-7a, lane 4: total RNA sample+10 pM let-7a, lane 5: total RNA sample + 20 pM let-7a. (b) Relationship between the pixel intensity and the additional concentration of synthetic let-7a in the final LCR reaction mixture.*

Table S1. The comparison between PCR, LCR, microarray, and blotting-based miRNA detection

Methods	Detection limit	Total RNA	Analysis time	Cost	Ref***
LCR assay	3.5 amol	40 ng	4 h	Low*	This work
Stem-loop RT-PCR	0.01 zmol	25 pg	6.5 h	High**	7a
LNA-blotting	1 fmol	2.5 μg	Over 48 h	High	6a
Microarray	0.2 amol	120 ng	Overnight	High	5c

Note: *The cost was calculated as about 2.2 USD per sample based on the selling price of the enzymes and DNA probes. **The cost was calculated as 7.5 USD per sample according to the selling price in China of the Kit for let-7a detection with stem-loop RT-PCR. Other costs were estimated based on the enzymes, DNA probes, and the instrumentation. ***Ref represents the cited references in the text of this paper.