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Electronic Supplementary Information

Ultrasensitive quantification of mature microRNAs by real-time PCR based on ligation of ribonucleotide-modified DNA probe

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Experimental Section

T4 RNA ligase 2 was purchased from New England Biolabs. HPLC-purified miRNAs, ribonucleotide-modified DNA probes, dNTPs, Ribonuclease inhibitor and DEPC-treated water were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). PAGE-purified DNA oligonucleotides were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The sequences of all RNA and DNA used were available in Table S1 and Table S2. The human lung total RNA sample was purchased from Ambion (USA). Taq Platinum DNA Polymerase was obtained from Tiangen Biotech Co., Ltd. (Beijing, China). The stem-loop RT-PCR assay kit with TaqMan probe for human let-7a detection was purchased from Applied Biosystems (USA). The real-time detection was performed according to the protocol offered by the supplier. All the solutions for ligation reactions were prepared in DEPC-treated water. All other reagents were of analytical reagent grade and used as purchased without further purification.

The ligation reaction mixture consisted of ligation buffer (50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 400 μM ATP), 1 U T4 RNA ligase 2, 2 nM probe A-M, 2 nM probe B, 20 U Ribonuclease inhibitor and appropriate amount of target miRNA or total RNA sample in a reaction volume of 10 μL. The probe A-M, probe B, and target miRNA were firstly mixed. The mixture was heated at 65 °C for 2 min and at 40 °C for 3 min. Then the ligase buffer and T4 RNA ligase 2 were added in the mixture, and the reaction mixture was incubated at 40 °C for 35 min to complete the ligation reaction. After the ligation reaction, the products were immediately put on ice.

A volume of 2 μL of the ligation product was transferred to PCR reaction mixture with final volume of 20 μL. The PCR reaction mixture included 1 U Taq Platinum DNA Polymerase, reaction buffer (20 mM Tris-HCl, pH 8.4, 10 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 20 mM KCl), 0.2 μM forward and reverse primer, 10 μM dNTPs, 0.2×SYBR Green□(Xiamen Bio-Vision Biotechnology, Xiamen, China), 1×ROX Reference Dye (Invitrogen, Beijing, China). The PCR reaction was carried out with a 7300 Real-Time PCR System (Applied Biosystems, USA) by using hot start of 94 °C for 5 min, followed by 40 cycles of 94 °C for 20 s and 60 °C for 30 s. The real-time fluorescence intensity was simultaneously monitored, which was calibrated by the fluorescence of ROX Reference Dye.

Table S1. The sequences of miRNAs and pre-let-7a miRNA.

	ID	sequense
miRNA	let-7a	5′–UGAGGUAGUAGGUUGUAUAGUU–3′
	let-7b	5′–UGAGGUAGUAGGUUGUGUGGUU–3′
	let-7c	5′–UGAGGUAGUAGGUUGUAUGGUU–3′
	let-7d	5′–AGAGGUAGUAGGUUGCAUAGU–3′
	let-7e	5′–UGAGGUAGGAGGUUGUAUAGU–3′
	let-7f	5′–UGAGGUAGUAGAUUGUAUAGUU–3′
	let-7g	5′–UGAGGUAGUAGUUUGUACAGU–3′
	let-7i	5′–UGAGGUAGUAGUUUGUGCUGU–3′
	mir-122	5′–UGGAGUGUGACAAUGGUGUUUG–3′
Pre-let-7a miRNA	let-7a-3	5′–GGGUGAGGUAGUAGGUUGUAU AGUUUGGGGCUCUGCCCUGCUAUGGG AUAACUAUACAAUCUACUGUCUUUCCU–3′

Table S2. The sequences of the DNA probes used for ligation reactions and the primers for PCR amplification.

probe A without modification	5′–GGGATACTGGAACCTGATGATGACTAACTAACTACCTGGCTGAT AACTATACAAC–3′
probe A-M modified with two ribonucleotides	5′–GGGATACTGGAACCTGATGATGACTAACTAACTACCTGGCTGAT AACTATACArArC–3′[α]
probe B	5′–PO_4-CTACTACCTCACCTTTGCTTTACTACTCTCACACACTCTATGCTTG CTACCGTCG–3′
probe A-M-122	5′–GGGATACTGGAACCTGATGATGACTAACTAACTACCTGACAAACACCA rUrU–3′[α]
probe B-122	5′–PO_4-GTCACACTCCAACTACTCTCACACACTCTATGCTTGCTACCGTCG–3′
universal forward primer for PCR amplification	5′–CGACGGTAGCAAGCATAGAGTGTG–3′
universal reverse primer for PCR amplification	5′–GGGATACTGGAACCTGATGATGAC–3′
forward primer for let-7a-3	5′–TAATACGACTCACTATAGGGAGGGTGAGGTAGTAGGTT **GTATAGTTTGGGGCTCTGCC**–3′[β]
reverse primer for let-7a-3	5′–AGGAAAGACAGTAGATTGTATAGTTATCCCATAGCAG **GGCAGAGCCCCAAACTATAC**–3′[β]

[α] The letter “r” indicates “ribonucleotide”.

[β] Let-7a-3 forward primer contains a portion of sequence complementary to let-7a-3 reverse primer. The complementary sequences were highlighted in the bold.

Effect of modification of DNA probe with ribonucleotides

As depicted in Figure S1a, by using probe A (see the sequence in Table S2) without any modification in the ligation reaction, the real-time fluorescence signals produced by let-7a in the concentration range from 0.2~20 fM can not efficiently separated from the blank. Let-7a can be detected only when its concentration is greater than 200 fM. We noticed that T4 RNA ligase 2 is generally used to ligate 3′-OH / 5′-PO_4 RNA nicks in either a duplex RNA or an RNA: DNA hybrid, but it is difficult to ligate DNA nicks.[S1] However, T4 RNA ligase 2 can efficiently catalyze the ligation between two ribonucleotides-modified DNA probe (at its 3′-terminus) and 5′-PO_4-modified DNA probe.[S2] Therefore, we design the probe A-M, which contains two ribonucleotides at its 3′-terminus, to perform the ligation reaction in the ligation-based PCR assay. As shown in Figure S1b, as low as 0.2 fM let-7a can be accurately detected by using the probe A-M in the ligation step and the C_T values are well linearly dependent on the logarithm (log) of let-7a concentrations in the range of 0.2 fM to 200 fM. The results indicate that the ligation efficiency of DNA probe A and probe B is very low templated by miRNA. The modification with two ribonucleotides at 3′-terminus of probe A (named probe A-M) can greatly increase the ligation efficiency by using miRNA as the template, and thus dramatically improve the sensitivity of miRNA detection. Therefore, probe A-M is utilized in the ligation-based PCR assay for miRNA detection.

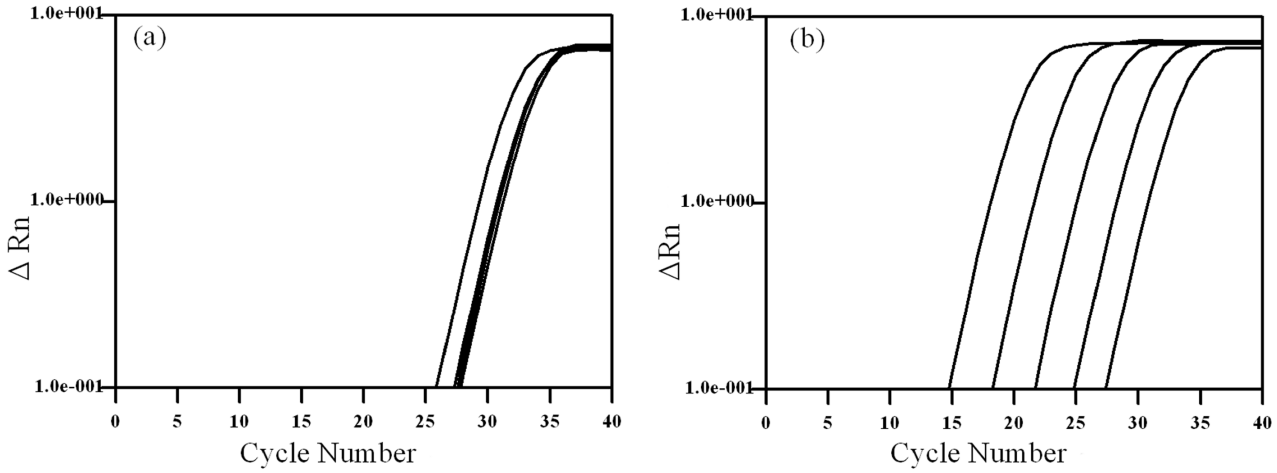

Fig. S1 Real-time fluorescence signals produced by synthetic let-7a miRNA in the ligation-based PCR assay respectively using probe A (a) and probe A-M (b) in the ligation step. Experimental conditions for the ligation reaction: [probe A] or [probe A-M] = 2 nM, [probe B] = 2 nM, [T4 RNA ligase 2] = 1 U. From right to left, the let-7a concentration successively is 0, 0.2, 2.0, 20, 200 fM. The ligation reactions were performed at 37 °C for 35 min. The PCR amplification and real-time fluorescence measurements were performed according to the experimental procedure.

Optimization of temperature for ligation reaction

The specificity of miRNA assay is very important due to the high similarity of miRNA sequences. For the ligation-based PCR assay, the specificity would be highly dependent on the selectivity of ligation reaction. The temperature for the ligation reaction is a critical factor to affect the selectivity. By respectively using let-7a and let-7b as the template, we perform the ligation reaction between probe A-M and probe B at 37 °C, 40 °C, 42 °C. After the PCR amplification, one can see from Figure S2 that the interference for let-7a detection arisen from the signal produced by equivalent amount of let-7b decreases with elevating the ligation temperature. However, the interference decreases little when the temperature is above 40 °C. On the other hand, the experiment data show that the ΔC_T between let-7a and the blank will be reduced with elevating the ligation temperature, and therefore, decreases the sensitivity of miRNA detection. Based on the consideration of both specificity and sensitivity, 40 °C was selected for the ligation reaction in the ligation-based miRNA assay.

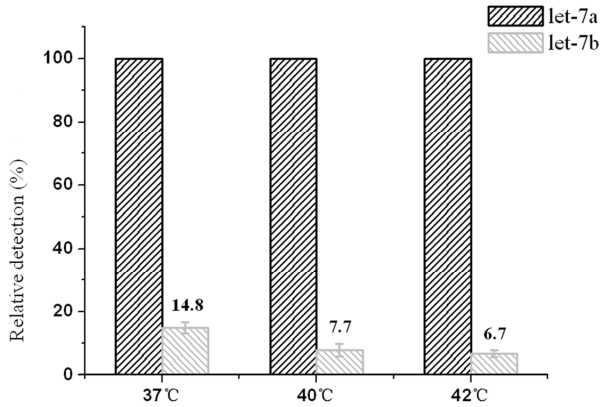

Fig. S2 Effect of ligation temperature on the specificity of miRNA assay. 2 pM let-7a and let-7b were respectively detected with the ligation-based PCR assay as described in the Experimental Section expect that the ligation reaction of probe A-M and probe B was performed at different temperature.

Preparation of pre-let-7a miRNA with *in vitro* transcription reaction and the specificity of the ligation-based PCR assay for detection of mature miRNA (let-7a) against pre-miRNA

Pre-let-7a miRNA (let-7a-3) is a precursor of let-7a miRNA, which is prepared by *in vitro* transcription reaction according to the previous literature.[S3] Firstly, let-7a-3 forward primer (FP) and reverse primer (RP) are designed, in which 20 bases in 3′-terminus of let-7a-3 FP is complementary to 20 bases in 3′-terminus of let-7a-3 RP (see the sequences in Table S2). 50 pmol of let-7a-3 FR and RP were mixed in a 10 μL volume of Klenow buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTT, pH 8.0). The mixture was incubated at 75 °C for 5 min and slowly cooled to room temperature (~30 min) to perform the hybridization between the 20 complementary bases. After that, dNTPs (250 μM final), 5 U Klenow DNA polymerase (exo-) (Fermentas, Shanghai, China), Klenow buffer and DEPC-treated deionized water were added in the mixture to give a final volume of 20 μL. With incubation at 37 °C for 1 h, the let-7a-3 FP and RP performed the extension reaction at their 3′-termini, respectively, to form a double stranded (ds) DNA. The reaction mixture was heated at 75 °C for 20 min to inactivate the Klenow DNA

polymerase and then slowly cooled to room temperature for dsDNA annealing. The dsDNA consisted of the T7 promoter, GGGA spacer and let-7a-3 specific sequence (from 5´to 3´-terminus in upper strand).

A volume of 20 μL of the dsDNA solution was added into 30 μL of *in vitro* transcription buffer (containing 2 mM NTPs, 40 mM Tris-HCl, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl, 2 mM spermidine, pH 7.9), 100 U Ribonuclease inhibitor, and 80 U T7 RNA polymerase (Fermentas, Shanghai, China). The *in vitro* transcription reaction was performed at 37 °C for 4 h to produce the pre-let-7a miRNA (let-7a-3). After that, the DNA in the reaction mixture was digested by adding 5 U of RNase-Free DNase I (TaKaRa, Dalian, China) and then the mixture was purified with EZ-10 spin column 5 minutes RNA cleanup & concentration kit (Bio Basic Inc., Shanghai, China). The final products of let-7a-3 were confirmed by electrophoresis analysis with 4% Agarose gel, and the concentration was determined from the absorption at 260 nm with TU-1901 UV-VIS Spectrophotometer (PGeneral, Beijing, China). The prepared pre-let-7a miRNA was used to investigate the specificity of the ligation-based PCR assay for detection of mature miRNA (let-7a) against pre-miRNA. The result was shown in Figure S3.

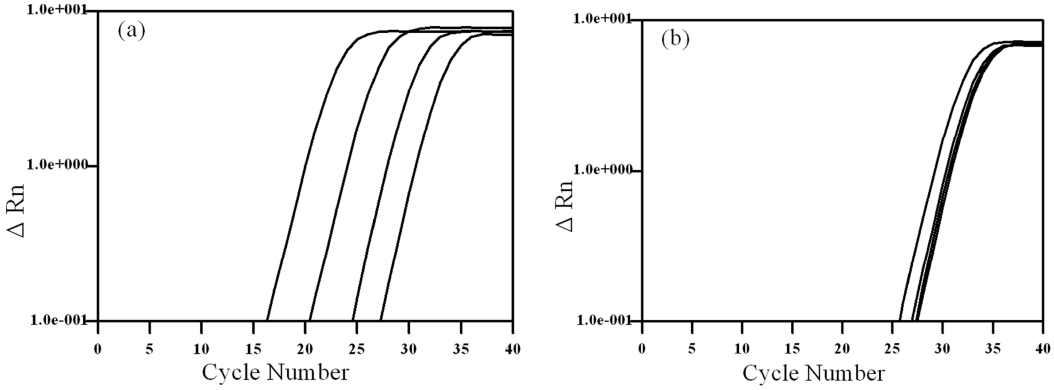

Fig. S3 Amplification plots of (a) let-7a and (b) let-7a-3 with the ligation-based PCR assay. From right to left in both (a) and (b), the concentrations of let-7a and let-7a-3 were respectively: 0, 2.0, 20, 200 fM.

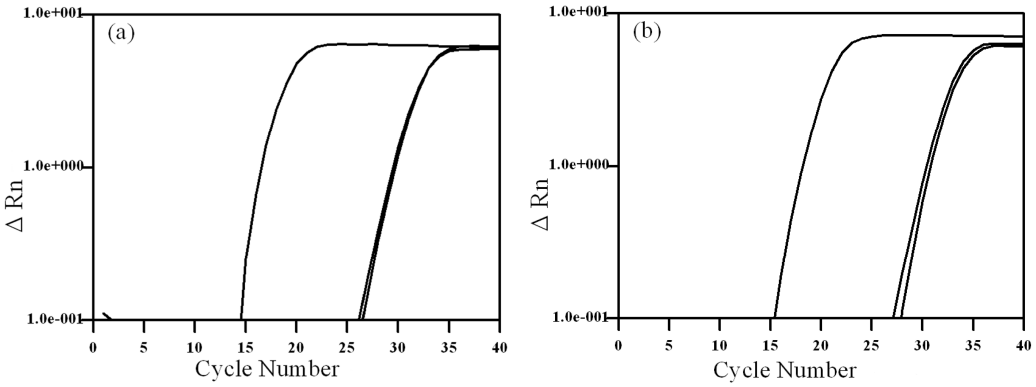

Fig. S4 Real-time detection of mir-122 and let-7a with the ligation-based PCR assay by using (a) mir-122-specific probes and (b) let-7a-specific probes. From right to left, (a): blank without miRNA, 2 pM let-7a, and 2 pM mir-122. (b): blank without miRNA, 2 pM mir-122, and 2 pM let-7a. The experimental conditions were the same as described in the Experimental Section.

Cost comparison among ligation-based PCR, ligase chain reaction (LCR), and stem-loop RT-PCR for miRNA detection

Ribonucleotide-modified DNA probes are relatively expensive than common DNA probes without ribonucleotide-modification (ribonucleotide-modified DNA probes are 31 USD/nmol; common DNA probes are 12 USD/nmol), but the usage amount is very little for each assay(2 nM in 10 μL= 2×10^{-5} nmol), therefore the use of ribonucleotide-modified DNA probes hardly increase the cost.

On the other hand, LCR is another microRNA detection method based on ligation of common DNA probes by using miRNAs as the templates, so we compare with it together. The LCR assay uses common DNA probes. However, the amount of T4 RNA ligase 2 used in the LCR assay is greater than that used in the ligation-based PCR assay (the ligased-based PCR used 1U and the LCR assay used 5U), which results in the higher cost per sample in LCR assay. In Table S3, the cost, time and sensitivity for miRNA detection are compared among ligation-based PCR, LCR and stem-loop RT PCR methods. The cost comparison proves that the proposed ligation-based PCR assay is very cost-effective.

Table S3. The comparison among ligation-based PCR, ligase chain reaction (LCR), and stem-loop RT-PCR for miRNA detection.

Methods	Detection limit	Analysis time	Cost	Ref***
Ligation-based PCR	4 zmol	2.5 h	Low*	This work
Stem-loop RT-PCR	0.01 zmol	6.5 h	Lower**	16
LCR assay	3.5 amol	4 h	High*	22

Note: *The cost per sample was about 1.2 USD for the ligation-based PCR assay and 2.2 USD for LCR assay, respectively, which was calculated 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. *** Ref represents the cited references in the text of this paper.

Signal generation of Background of NTC and the blank

Fig. S5 shows the electrophoresis analysis results of the NTC, blank and the ligation-based PCR product. One can see from Fig. S5 that NTC has a by-product with about 50 bp, which can presumably be ascribed to the formation of primer-dimer. In this work, we use SYBR Green I as the fluorescent dye for real-time detection of PCR products. SYBR Green I has high affinity to all double-stranded DNA (dsDNA), and thus, can not differentiate the by-products from the specific product of PCR. Therefore, as shown in Fig. 2a, the background of NTC in the ligation-based PCR assay is very high in comparison with the data by stem-loop RT-PCR, in which the specific TaqMan probe is used to detect the PCR product. Except the by-product with about 50 bp, the blank produced by 0 fM let-7a shows another by-product with about 80 bp, which is generally associated with non-specific amplification of PCR. As a result, one can see from Fig. 2 that there is a significant difference in cycle number between the curve “NTC” and curve “0 fM let-7a”. On the other hand, the experiment results

described above indicate that the background of NTC and the blank signal of 0 fM let-7a should be greatly decreased if the specific TaqMan probe is used to detect the ligation-based PCR products, and thus, the sensitivity for miRNA detection can be probably improved by using the specific TaqMan probe.

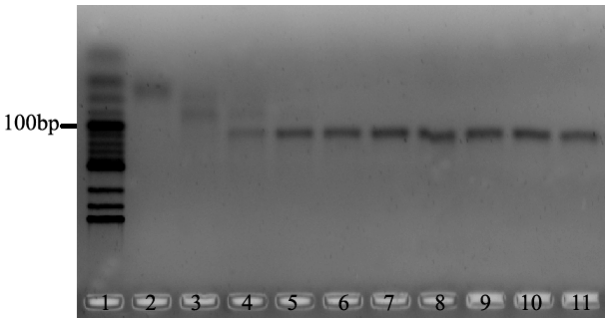

Fig. S5 The electrophoresis analysis with 4% agarose gel of the ligation-based PCR assay: lane 1: DNA ladder marker, lane 2: NTC, lanes 3-11: the concentration of let-7a successively is 0, 0.2 fM, 2 fM, 20 fM, 200 fM, 2 pM, 20 pM, 200 pM, 2 nM.

References:

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S3. J. Li, B. Yao, H. Huang, Z. Wang, C. Sun, Y. Fan, Q. Chang, S. Li, X. Wang and J. Xi, *Anal. Chem.*, 2009, **81**, 5446–5451.