Electronic Supplementary Information

Sensitive quantification of messenger RNA with real-time ligase chain reaction by using a ribonucleotide-modified DNA probe

Yanlei Hu, Hongxia Jia, Yucong Wang, Yongqiang Cheng, Zhengping Li*

Key Laboratory of Medicinal Chemistry and Molecular Diagnosis, Ministry of Education, College of Chemistry and Environmental Science, Hebei University, Baoding 071002, Hebei Province, P. R. China.

List of Contents:

Supporting Information

1. Materials and reagents

T4 RNA Ligase 2 and *Taq* DNA ligase were purchased from New England BioLabs (USA). All the synthetic RNA and DNA, Ribonuclease Inhibitor and DEPC-treated water were obtained from TAKARA Biotechnology Co., Ltd. (Dalian, China). The sequences of all the synthetic RNA and DNA were listed in Table S1. All the oligonucleotide concentrations were verified by the absorbance at 260 nm. SYBR Green I (20 × stock solution in dimethyl sulfoxide) was purchased from Xiamen Bio-vision Biotechnology (Xiamen, China). StepOne Real-Time PCR System (Applied Biosystems, USA) was employed for real-time fluorescence measurement. A 2720 Thermal Cycler (Applied Biosystems, USA) was used to control the reaction temperature. TU-1901 UV-VIS spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China) was used to quantify the nucleic acid concentrations.

Table S1. The sequences of the synthetic mRNA target, variant mRNA and the DNA probes used in the LCR.

Name	Sequence
mRNA target	5′–UCAUUCCAAAUAUGAGAUGCGUUGUUACAGGAAGUCCCUU–3′[α]
variant mRNA target	5′–UCAUUCCAAAUAUGAGAUGCAUUGUUACAGGAAGUCCCUU – 3′[α]
probe A	5′–PO_4-GCATCTCATATTTGGAA–3′
probe B	5′–GACTTCCTGTAACAAC–3′
probe B-M	5′–GACTTCCTGTAACArArC–3′[β]
probe C	5′–TTCCAAATATGAGATGC–3′
probe D	5′–PO_4-GTTGTTACAGGAAGTC–3′

[α] The one-nucleotide variation between mRNA and variant mRNA is underlined.

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

2. Total RNA extraction

Hela cells was obtained from Hebei Medical University and cultured in RPMI 1640 (GIBCO, Cat. 31800-022), which contains 10% (v/v) fetal bovine serum, 0.2% $NaHCO_3$, 100 U/mL penicillin, 100 μg/mL streptomycin and 3 mmol/L L-glutamine. Total RNA was extracted from

the cells using Trizol® Reagent (Invitrogen, Beijing, China) following the manufacturer's protocol. The concentration was determined by the absorption at 260 nm with a TU-1901 UV-VIS spectrophotometer.

3. Standard experimental procedures of LCR-based mRNA assay

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, 5 nM probe A, 5 nM probe B-M, 8 U Ribonuclease inhibitor and appropriate amount of target mRNA or total RNA sample in a reaction volume of 10 μL. Probe A, probe B-M, ligase buffer and target mRNA were firstly mixed. The mixture was heated at 90 °C for 1 min and at 4 °C for 1 min. Then T4 RNA ligase 2 and Ribonuclease inhibitor were added in the mixture, and the reaction mixture was incubated at 40 °C for 30 min to complete the ligation reaction. After the ligation reaction, the products were immediately put on ice.

A volume of 1 μL of the ligation product was transferred to 10 μL of the LCR reaction mixture containing probe A, probe B, probe C, probe D (each concentration was 50 nM), 10 U Taq DNA ligase, the reaction buffer (20 mM Tris-HCl, pH 7.6, 25 mM KAc, 10 mM $Mg(Ac)_2$, 10 mM DTT, 1 mM NAD, 0.1% Triton X-100) and 1×SYBR Green I. The reaction mixture was firstly heated at 80 °C for 20 s, then performed 50 thermal cycles: 80 °C for 10 s, 45 °C for 30s and 65 °C for 10s with StepOne Real-Time PCR System. The real-time fluorescence intensity was monitored at 65°C in every thermal cycle.

4. Description of the thermal cycles in LCR

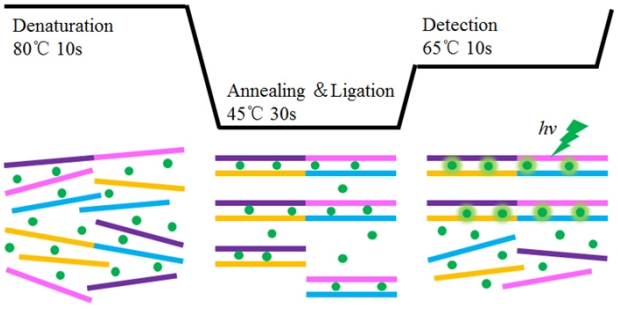

Fig. S1 Schematic description of the thermal cycles in the LCR amplification and real-time fluorescence measurement.

5. Influence of ligation temperature in the first ligation step

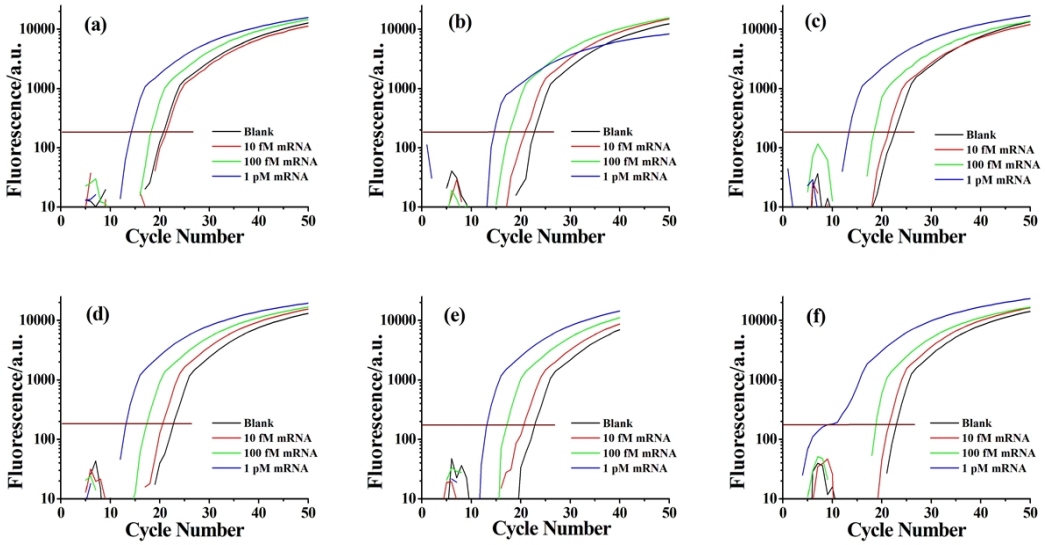

Fig. S2 Real-time fluorescence curves produced by the synthetic mRNA target with different concentration (from right to left, the mRNA concentration successively is 0, 10 fM, 100 fM and 1 pM) at different ligation temperature at the first ligation step: (a) 37°C, (b) 38°C, (c) 39°C, (d) 40°C, (e) 41°C, (f) 42°C. Experimental conditions in the first ligation reaction: [probe A] = [probe B-M] = 10 nM, [T4 RNA Ligase 2] = 1 U. Experimental conditions in the LCR: [probe A] = [probe B] = [probe C] = [probe D] = 100 nM and [*Taq* DNA ligase] = 10 U. The first ligation reaction was performed for 30 min at different temperature. The LCR was performed with 50 thermal cycles: 80°C for 10s, 45°C for 30s and 65°C for 10s. The fluorescence intensity of LCR products was monitored at 65°C in every thermal cycle.

To investigate the influence of the temperature at the first ligation step, the ligation reaction was performed at 37°C ~ 42°C and the real-time fluorescence signals produced by the synthetic mRNA target with the LCR-based mRNA assay were monitored. As shown in Fig. S2, when the ligation reaction was performed at 37°C, the *Ct* value of blank is small and the real-time fluorescence signals respectively produced by blank and 10 fM mRNA target can not be separated, indicating high blank signal and low sensitivity of the mRNA assay, which may be attributed to the seriously non-specific ligation at low temperature. With elevating the ligation temperature, the real-time fluorescence signals between the 10 fM mRNA target and blank can be gradually separated and the difference of the corresponding *Ct* values achieves maximum when the ligation temperature is at 40°C and 41°C. When the ligation temperature is elevated to 42°C, the *Ct* value

of blank becomes large and the difference of *Ct* values between 10fM mRNA target and the blank decreases, probably because the activity of T4 RNA ligase 2 can be decreased at higher temperature, leading to low efficiency of ligation of DNA probes in the first ligation step. Therefore, 40°C was selected as the optimized ligation temperature in the first ligation step for the LCR-based mRNA assay.

6. Effect of the concentration of probe A and probe B-M

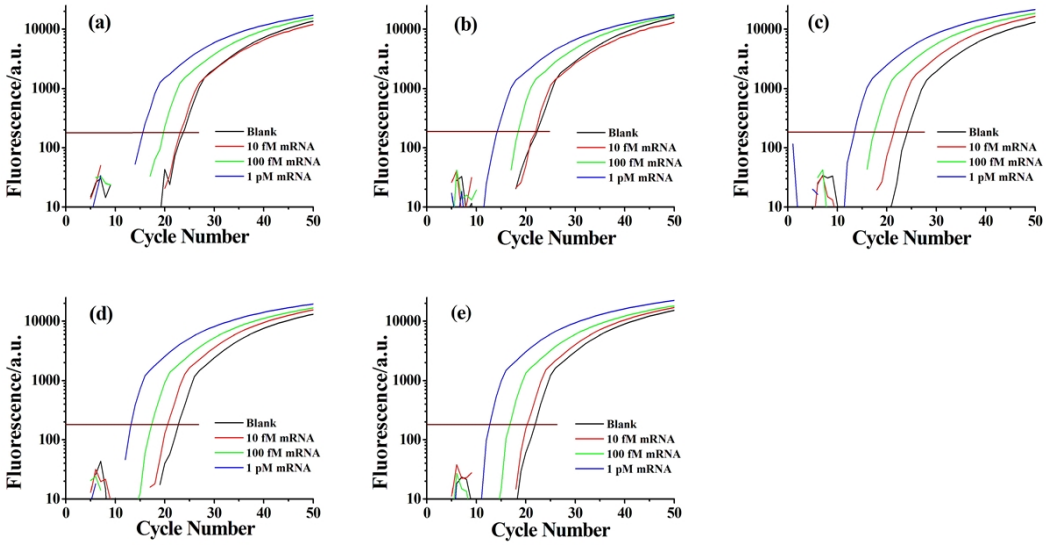

Fig. S3 Real-time fluorescence curves produced by the synthetic mRNA target with different concentration (from right to left, the mRNA concentration successively is 0, 10 fM, 100 fM and 1 pM) by using (a) 1 nM, (b) 2 nM, (c) 5 nM, (d) 10 nM and (e) 100 nM of probe A and probe B-M respectively. Experimental conditions: [T4 RNA Ligase 2] = 1 U in the first step; [probe A] = [probe B] = [probe C] = [probe D] = 100 nM and [*Taq* DNA ligase] = 10 U in LCR. The ligation reaction in the first step was performed at 40°C for 30 min. The thermal cycles of 80°C for 10s, 45°C for 30s and 65°C for 10s was performed in LCR. The fluorescence intensity of LCR products was monitored at 65°C in every thermal cycle.

As described above, the ligation of probe A and probe B-M templated by mRNA is a critical factor to affect the LCR-based mRNA assay, because the ligation product of AMB (see Fig. 1) is a milestone to initial the following LCR amplification. We investigated the effect of the concentration of probe A and probe B-M on the LCR-based mRNA assay by using probe A and probe B-M with different concentration in the range of 1 nM ~ 100 nM. As depicted in Fig. S3, the real-time fluorescence signals between blank and 10 fM mRNA target can not be clearly

discriminated when the concentration of probe A and probe B-M was less than 2 nM (Fig. S3a and S3b). The results should be attributed to that the low concentration of probe A and probe B-M can result in low ligation efficiency, leading to low sensitivity of the LCR-based mRNA assay. On the other hand, when the concentration of probe A and probe B-M was greater than 10 nM (Fig. S3 d and e), the *Ct* value of blank signal decreased with increasing the concentration of probe A and probe B-M, resulting in reduced different of *Ct* values between blank and 10 fM mRNA target. The too excessive probe A and probe B-M could cause much template-independent ligation, inducing non-specific LCR amplification and large blank signal. When 5 nM of probe A and probe B-M was used for the ligation reaction, as shown in Figure S3c, the difference of *Ct* values between blank and 10 fM mRNA target achieved its maximum, indicating that 5 nM of probe A and probe B-M was appropriate. Therefore, 5 nM of probe A and probe B-M was used for the subsequent work.

7. Effect of the concentration of probe A, B, C, and D in LCR

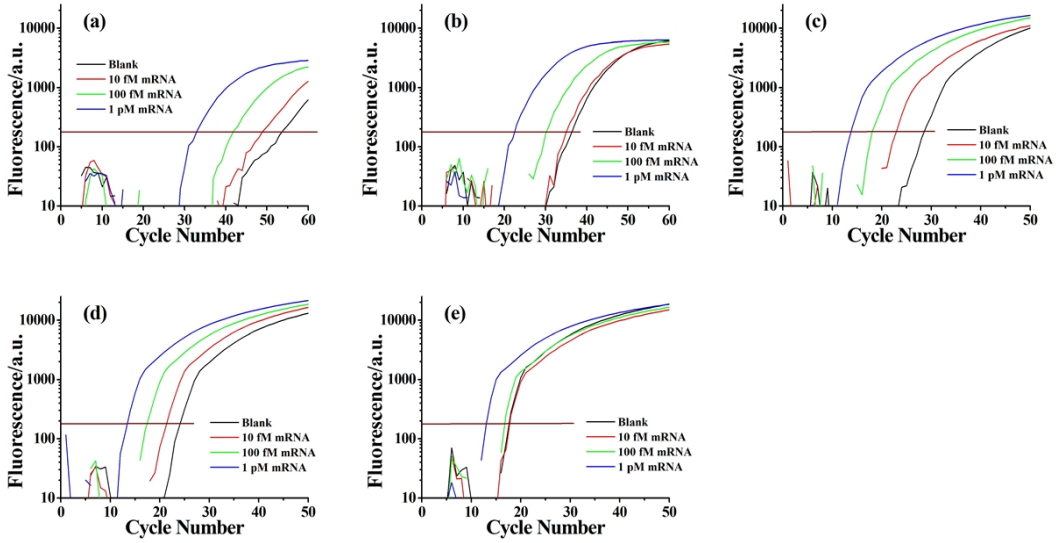

Fig. S4 Real-time fluorescence curves produced by the synthetic mRNA target with different concentration by using (a) 10 nM, (b) 20 nM, (c) 50 nM, (d) 100 nM and (e) 200 nM of probe A, probe B, probe C, and probe D respectively. Experimental conditions: [probe A] = [probe B-M] = 5 nM and [T4 RNA Ligase 2] = 1 U in the first step; [*Taq* DNA ligase] = 10 U in LCR. The ligation reaction in the first step was performed at at 40°C for 30 min. The thermal cycles of 80°C for 10s, 45°C for 30s and 65°C for 10s was performed in LCR. The fluorescence intensity of LCR products was monitored at 65°C in every thermal cycle.

To investigate the effect of the concentration of probe A, B, C, and D used in LCR on the LCR-based mRNA assay, different concentration ranged from 10 M to 200 nM of probe A, B, C, and D was used to perform the LCR-based mRNA assay. As shown in Fig. S4 a, the real-time fluorescence signals were weak and unstable when 10 nM of probe A, B, C, and D was used in LCR. By using 20 nM of probe A, B, C, and D, one can see from Fig. S4 b that the real-time fluorescence signals was greatly enhanced, but the difference of *Ct* values between blank and 10 fM mRNA target was very small, indicating the low sensitivity of the LCR-based mRNA assay. On the other hand, when the concentration of probe A, B, C, and D used in LCR was greater than 100 nM (Fig. S4 d and e), the difference of *Ct* values between blank and 10 fM mRNA target would be decreased with increasing the concentration of probe A, B, C, and D, as described above, because too excessive DNA probes could cause the template-independent ligation, inducing non-specific LCR amplification. As shown in Fig. S4 c, when 50 nM of probe A, B, C, and D was used in the LCR, the difference of *Ct* values between blank and 10 fM mRNA target reached its maximum. Therefore, 5 nM was selected as the optimized concentration of probe A, B, C, and D for the LCR.

8. Effect of the amount of *Taq* DNA ligase on the mRNA assay

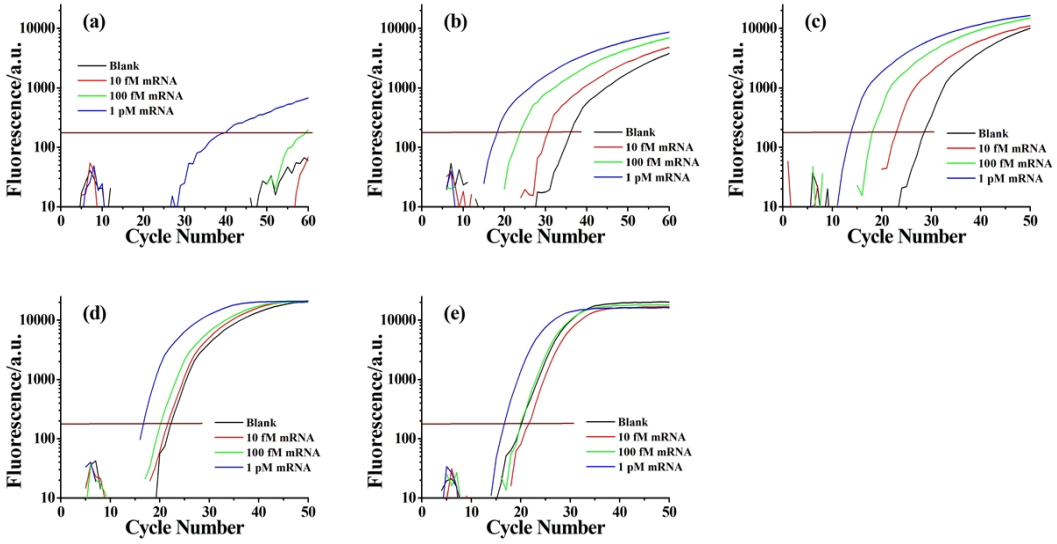

Fig. S5 Real-time fluorescence curves produced by the synthetic mRNA target with different amount of *Taq* DNA ligase (a) 1 U, (b) 2 U, (c) 5 U, (d) 10 U and (e) 20 U and (f) 40 U. Experimental conditions: [probe A] = [probe B-M] = 5 nM and [T4 RNA Ligase 2] = 1 U in the first step; [probe A] = [probe B] = [probe C] = [probe D] = 50 nM in LCR. The ligation reaction in the first step was performed at 40°C for 30 min. The thermal cycles of 80°C for 10s, 45°C for 30s and 65°C for 10s was performed in LCR. The fluorescence intensity of LCR products was

monitored at 65°C in every thermal cycle.

To study the effect of the amount of *Taq* DNA ligase used in LCR on the LCR-based mRNA assay, the different amount of *Taq* DNA ligase ranged from 2 U to 40 U was used in the LCR. The trend of the effect of the amount of *Taq* DNA ligase is similar to that of the concentration of DNA probes described above. As demonstrated in Fig. S5 a, when the amount of *Taq* DNA ligase used in the LCR small (1 U), the real-time fluorescence signals produced in the LCR-based mRNA assay is very low and unstable. On the other hand, when the amount of *Taq* DNA ligase used in the LCR was greater than 20 U (Fig. S5 d and e), the excessive amount of *Taq* DNA ligase resulted in non-specific LCR amplification, which would reduce the difference of *Ct* values between blank and mRNA target and lead to low sensitivity for mRNA detection. As shown in Fig. S5 c, when 10 U *Taq* DNA ligase was used in the LCR, the difference of *Ct* values between blank and mRNA target could be obtained its maximum. Therefore, 10 U *Taq* DNA ligase was chosen as the optimized amount for the LCR-based mRNA assay.

9. Calculation of the relative detection

The mRNA target and the variant mRNA were simultaneously detected with the proposed mRNA assay. The real-time fluorescence curves produced by 100 fM mRNA target and the variant mRNA were shown in Fig. 5. We supposed the *Ct* values corresponding to the real-time fluorescence curves of the mRNA target and the variant mRNA were Ct_1 and Ct_2, respectively. According to the correlation equation obtained from Fig. 4, the Ct_1 and Ct_2 were corresponding to the concentration of the mRNA target as *C1* and *C2*, respectively. The relative detection of the mRNA target was normalized to 100%. The relative detection of the variant mRNA was defined as $\frac{C2}{C1}\times 100\%$, which was used to represent the interference of the variant mRNA for the detection of the mRNA target and to evaluate the specificity of the mRNA assay.

The correlation equation obtained from Fig. 4 was as follows:

$$C_t = -44.69 - 5.05\lg C_{tmRNA} \qquad (1)$$

According to the correlation equation, the $\frac{C2}{C1}$ can be obtained as follows:

$$\lg\frac{C2}{C1} = -\frac{C_{t2} - C_{t1}}{5.05} \qquad (2)$$

According to the equation (2) and the average value of Ct_1 and Ct_2 as shown in Fig. 5a, the

relative detection of the variant mRNA $\frac{C2}{C1}\times 100\%$ was calculated to be 18.6%.

10. Determination of β-actin mRNA in total RNA sample

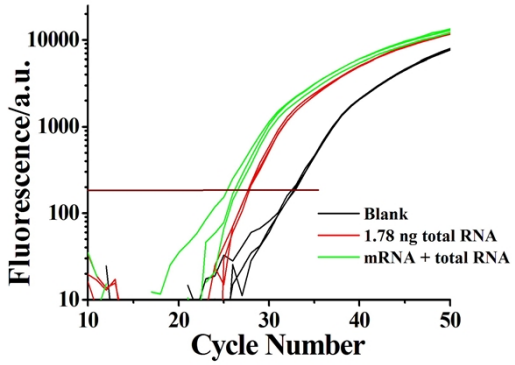

Fig. S6 The real-time fluorescence curves for determination of β-actin mRNA in total RNA samples.