Supporting Information

Direct recognition and sensitive detection of circular RNA with ligation-based PCR

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1. Materials and reagents.

SplintR ligase was purchased from New England BioLabs (U.S.A.). Hot Start Taq DNA polymerase, 10×PCR Buffer (Mg^{2+} plus), RNase-free water, Recombinant RNase Inhibitors (RRI) and deoxy-ribonucleoside triphosphate solution (dNTPs) were obtained from TaKaRa (Dalian, China). SYBR Green I (20 ng/μL, DMSO as solvent) was purchased from Xiamen Bio-Vision Biotechnology (Xiamen, China). Trizol reagent and lipofectamine 3000 were obtained from Invitrogen (Beijing, China). The 293T cells were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). The reagents used in the experiments were all analytical pure, and used as received without further purification. CDR1AS (hsa_circ_0001946) gene fragment was synthesized by Chemgenes (U.S.A.). HPLC-purified DNA strands consisting of normal probe, 5'-terminal phosphorylated DNA probe and PCR primers were synthesized by Nanjing Kingsray Biotechnology Co., Ltd. The sequences of the oligonucleotides were shown in Table S1 and S3. Quanstudio 3 Real-Time quantitative PCR system (Applied Biosystems, U.S.A.) was used to monitor the fluorescence signal.

Table1. The sequences used in the experiment.

Name	Sequences (5'-3' direction)
CDR1AS fragment	GGCTCAATATCCATGTCTTCCAACGTCTCCAGTG TGCTGATCTTCTGACATTCAGGTCTTCCAGTGTCT GCAATATCCAGGGTTTCCGATGGCACCTGTG
Linear RNA 1	GGCTCAATATCCATGTCTTCCAACGTCTCCAGTG TGCTGATCTTCTGACATTCAGGTCTTCCAGTGTCT GCAATATCCAG
Linear RNA 2	GGTTTCCGATGGCACCTGTGTCAAGGTCTTCCAA CAACTCCGGGTCTTCC
Probe A	Phosphate-CTGGATATTGCAGACACTGGCTCTATGGGCAGTC GGTGAT
Probe B	CCATCTCATCCCTGCGTGTCCACAGGTGCCATCG GAAACC
Forward primer	ATCACCGACTGCCCATAGAG
Reverse primer	CCATCTCATCCCTGCGTGTC

2. Transfection of 293T cells.

The 293T cells were cultivated under a humid atmosphere containing 5% CO_2 at 37 ^oC. The culture medium included DMEM medium supplemented with 10% (v/v) fetal calf serum, 100 μg/mL streptomycin and 100 U/mL penicillin. Then 293T cells were divided into three groups: the first group was transfected with the CDR1AS overexpression plasmid (C1946 plasmid), the second group was transfected with the empty vector plasmid (pLCDH-ciR) and the last group was 293T cells without any treatment which acted as negative control. Transfections were conducted using lipofectamine 3000 according to the protocol. Total RNA was extracted from those cells using Trizol reagent according to the procedure. The concentration of total RNA extracted from cells was quantified using Nanodrop One.

3. Ligation of DNA probes.

The ligation reaction mixture included 1 nM probe A, 1 nM probe B, 8 U RRI, 1 × SplintR ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, pH 7.5@ 25 ^oC) and different amounts of CDR1AS fragment or total RNA samples. The mixture was heated at 65 ^oC for 5 min and 37 ^oC for 5 min. Then 5 U SplintR ligase was added in the mixture. The reaction mixture was incubated at 37 ^oC for 20 min to complete the ligation reaction. After the ligation reaction, the products were put on ice right away.

4. PCR amplification.

The reaction mixture consisted of 0.25 mM dNTPs, 1 × PCR Buffer (Mg^{2+} plus), 0.2 μM forward primer, 0.2 μM reverse primer, 0.4 ng/μL SYBR Green I, 1 U Hot Start Taq DNA polymerase and 1 μL ligation product. The mixture was put into the Quanstudio 3 Real-Time quantitative PCR system to carry out the PCR reaction with following procedure: 95 ^oC for 4 min to start the reaction, then procedure of 95 ^oC for 20 s, 65 ^oC for 30 s, 72 ^oC for 20 s for 40 cycles. The fluorescence signal value was detected at 72 ^oC.

5. Optimization of the concentration of DNA probes during ligation step.

The concentration of DNA probes was an important factor to affect the ligation efficiency due to the ability to form the ligation products which were the template of

PCR. So the amount of DNA probes during ligation step was optimized from the concentration of 500 pM to 5 nM. The optimal concentration of DNA probes was investigated by detecting the synthetic circRNA target at 0, 10 fM and 100 fM, respectively. As shown in Figure S1, when the concentration of DNA probes was 500 pM, the ligation efficiency was relatively low as the Ct value between 100 fM and blank was small. Increasing the concentration of DNA probes to 1 nM, the difference between 100 fM and blank increased, demonstrating that the ligation efficiency increased along with elevated concentration of DNA probes. Further increasing the concentration of DNA probes to 5 nM, non-specific ligation and corresponding non-specific amplification became obvious along with the increased ligation efficiency, leading to the inseparable fluorescence curves produced by 10 fM target and blank. In consideration of both the ligation efficiency and non-specific amplification, 1 nM was selected as the optimized concentration of DNA probes in the first ligation step.

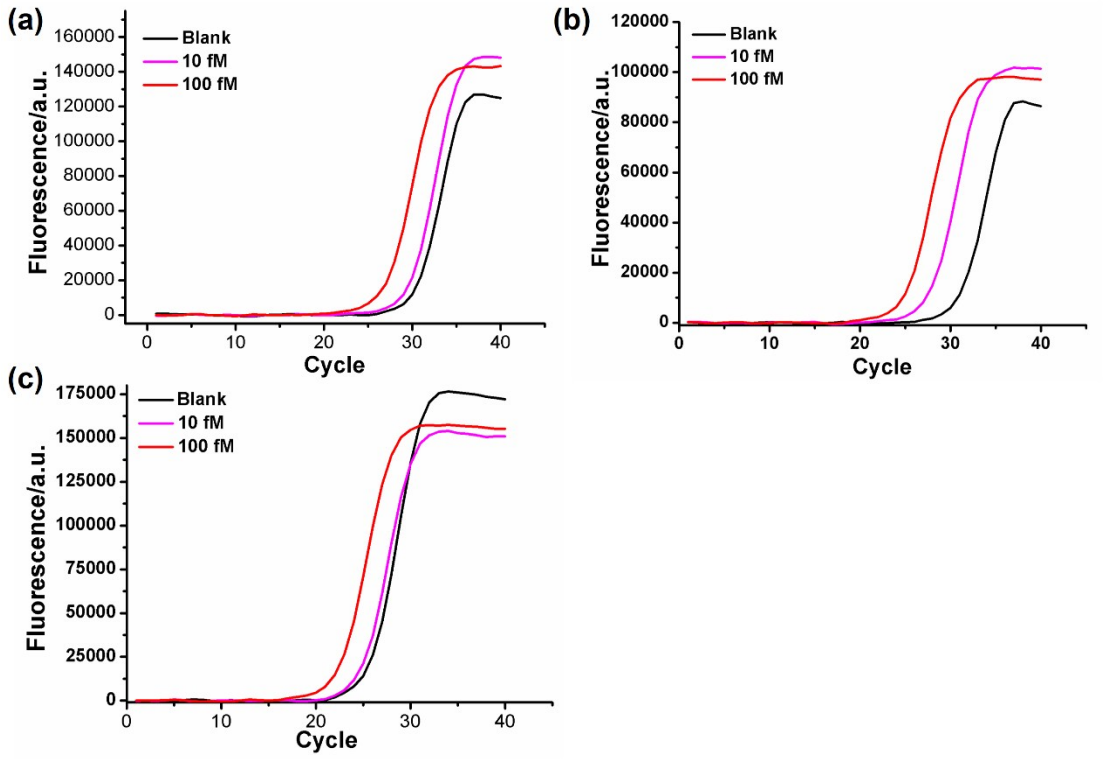

Figure S1. Real-time fluorescence curves produced by the synthetic circRNA target with different concentrations of DNA probes. The concentration of DNA probes was 500 pM (a), 1 nM (b) and 5 nM (c), respectively.

6. Optimization of the temperature during ligation step.

The ligation temperature determined the catalytic activity of SplintR ligase, therefore, the ligation temperature was optimized. According to the specification, the activity of splintR ligase is optimum at 16 °C -37 °C. The lower the temperature, the higher the nonspecific hybridization. The ligation temperature was investigated by detecting synthetic circRNA target at 0, 10 fM and 100 fM with the temperature ranging from 28 °C to 39 °C, respectively. As shown in Figure S2, the difference of Ct value between 10 fM and blank increased along with increased ligation temperature from 28 °C-37 °C, demonstrating that the ligation efficiency increased within this range. When the ligation temperature increased to 39 °C, the difference of Ct value between 10 fM and blank decreased, which meant that 39 °C was too high for splintR ligase. Therefore, 37 °C was chosen as the optimum ligation temperature.

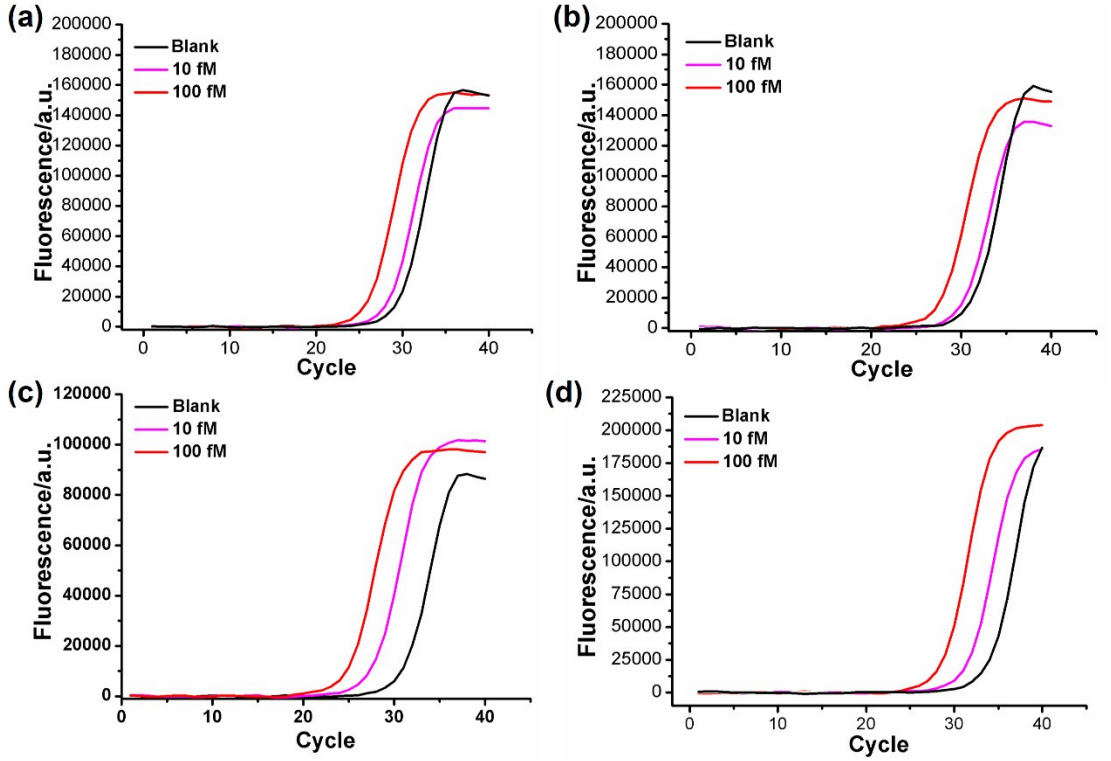

Figure S2. Real-time fluorescence curves produced by the synthetic circRNA target with different ligation temperature. The temperature for the ligation reaction was 28 °C (a), 33 °C (b), 37 °C (c) and 39 °C (d), respectively.

7. Optimization of annealing temperature during the PCR step.

The annealing temperature of PCR might affect the amplification efficiency of ligation products. So the effect of PCR annealing temperature was investigated and optimized, following the procedures mentioned above except that the annealing temperature ranging from 60 °C to 68 °C. As displayed in Figure S3, when 60 °C was used, the fluorescence curve produced by 1 fM circRNA was almost the same with that of blank which did not have circRNA, demonstrating that the nonspecific amplification was relatively high at lower temperature. When the annealing temperature increased to 65 °C, the nonspecific amplification decreased apparently and the fluorescence curve produced by 1 fM circRNA could be well separated with that of blank. Further increasing the annealing temperature to 68 °C, the fluorescence curve produced by 1 fM circRNA retreated to that of blank, which might lead to lower sensitivity for circRNA detection. According to the results depicted in Figure S3, 65 °C was chosen as the optimal PCR annealing temperature.

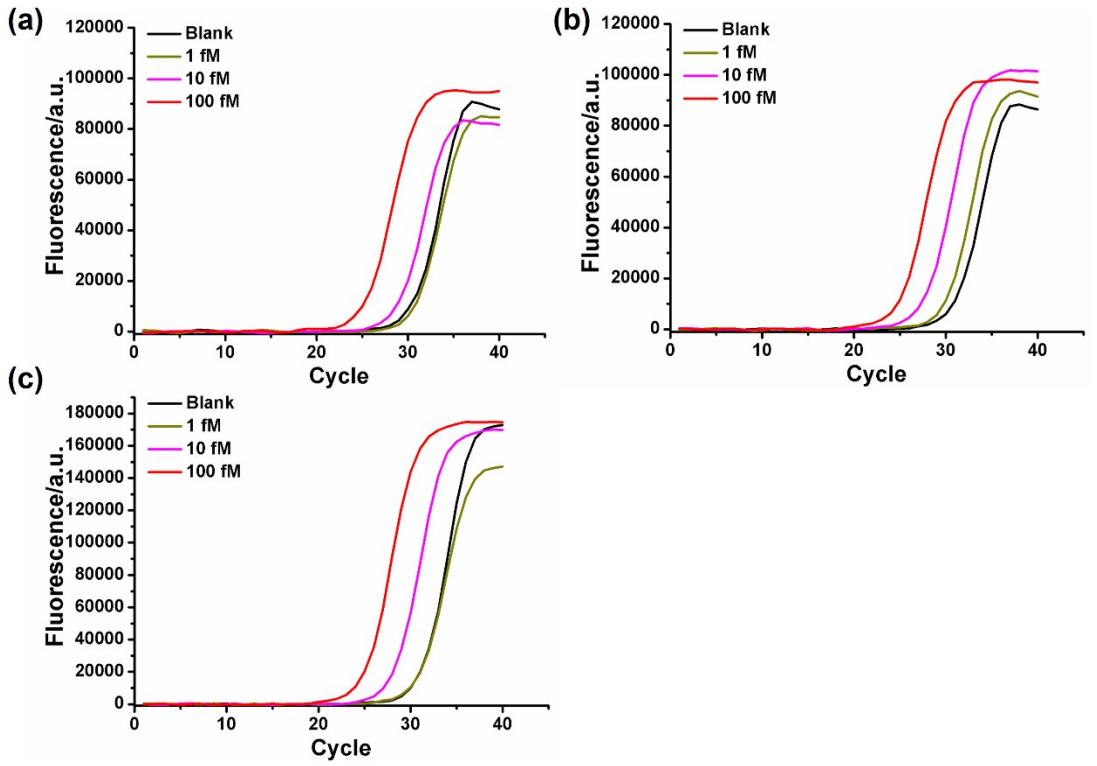

Figure S3. Real-time fluorescence curves produced by the synthetic circRNA target with different annealing temperatures. The annealing temperature was 60 °C (a), 65 °C (b) and 68 °C (c), respectively.

8. The relative expression of CDR1AS in total RNA extracted from 293T cells after transfection with different plasmids.

Firstly, total RNA was extracted from 293T cells after transfected with different plasmids and undergone the reverse transcription reaction using random primer. The reverse transcription mixture consisted of appropriate amount of total RNA, 0.25 mM dNTPs, 1 μM random primer, 4 U RRI, 20 U ProtoScript II reverse transcriptase, 50 mM Tris-HCl and 3 mM $MgCl_2$ (pH 8.3 @ 25 °C). The solution was incubated at 16 °C for 30 min, 42 °C for 30 min and then 95 °C for 5 min to inactive ProtoScript II reverse transcriptase. Then the reverse transcription product was added into the PCR mixture containing 0.5 μM divergent primers (Table S3), 0.25 mM dNTPs, 1 × PCR buffer and 1 U Hot Start Taq DNA polymerase. The divergent primers, which were used to amplify circRNA specifically, were designed to be divergent to the junction site of CDR1AS. The PCR mixture was then incubated at 95 °C for 4 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Table S2. The relative expression of CDR1AS in total RNA extracted from 293T cells.

Sample Name	GAPDH Average Ct	CDR1AS Average Ct	Δ CT	Δ Δ CT	$2^{-\Delta\Delta CT}$
293T control	20.87±0.03	23.78±0.02	2.91±0.04	0±0.04	1
293T with PLCDH plasmid	20.61±0.04	23.77±0.16	3.17±0.16	0.26±0.16	0.83
293T with C1946 plasmid	20.72±0.05	16.66±0.01	-4.06±0.05	-6.96±0.05	124.7

9. Sequencing results of the CDR1AS junction site in total RNA extracted from 293T cells after transfected with the CDR1AS overexpression plasmid.

The procedure was the same with the relative expression of CDR1AS in total RNA extracted from 293T cells after transfection with different plasmids, except that the PCR products was sequenced by Sangon Biotech (Shanghai, China) using sequencing primers.

Table S3. The sequences of the oligonucleotides used in the verification step.

Name	Sequence (5'-3')
CDR1AS	…CTTCCAGCATCTCTGTGTCTTCCAGCATC TTCATGTCTTCCAACAACTACCCAGTCTTC CATCAACTGGCTCAATATCCATGTCTTCCA ACGTCTCCAGTGTGCTGATCTTCTGACATT CAGGTCTTCCAGTGTCTGCAATATCCAG--- GGTTTCCGATGGCACCTGTGTCAAGGTCTT CCAACAACTCCGGGTCTTCCAGCGACTTC AAGTCTTCCAATAATCTCAAGGTCTTCCAG ATAATCCTGAGCTTCCAGAAAATCCACAT CTTCCAGACAATCCATGTCTTCCGGAC…
Forward primer for GAPDH	GCTGAGAACGGGAAGCTTGT
Reverse primer for GAPDH	GACTCCACGACGTACTCAGC
Divergent former primer	GTCTTCCAACAACTACCCAGTC
Divergent reverse primer	GTGCCATCGGAAACCCTGGA
Sequencing Forward Primer	GTCTTCCAACAACTACCCAGTC
Sequencing Reverse Primer	TGGATTTTCTGGAAGCTCAGG

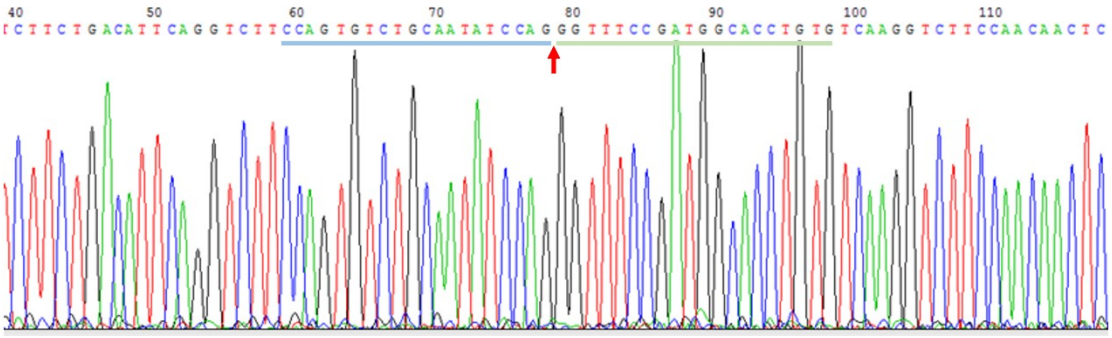

Figure S4. The sequencing result of the CDR1AS junction site in total RNA extracted from 293T cells after transfected with the CDR1AS overexpression plasmid. The blue line indicated the recognition sequence of probe B and the green line indicated the recognition sequence of probe A in the proposed assay. The red arrow indicated the

junction site of CDR1AS.

The sequencing result shown in Figure S6 indicated that total RNA extracted from 293T cells after transfected with the CDR1AS overexpression plasmid had the CDR1AS junction site, demonstrating the existence of CDR1AS which was in accordance with our results.