Electronic Supplementary Information

Multiple Stem-loop Primers Induced Cascaded Loop-Mediated Isothermal Amplification for Direct Recognition and Specific Detection of Circular RNA

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1. The synthesis of CDR1AS fragments

The CDR1AS fragments were synthesized by transcription of DNA template containing T7 promoter. The DNA template was prepared by PCR using two primers as shown in Table S1. The PCR reaction mixture consisted of 0.25 mM dNTPs, 1 × PCR buffer (Mg^{2+} plus), 0.5 μM forward primer, 0.5 μM reverse primer, 1 U Taq Hot Start DNA polymerase and 1 pM T7 DNA template. PCR reaction was carried out with following procedure: 95 °C for 4 min to start the reaction, then procedures of 95 °C for 20 s, 60 °C for 30 s, 72 °C for 20 s for 40 cycles. After that, the reaction was carried out at 72 °C for 7min. After purification, the concentration of PCR products was determined using Nanodrop One.

For the transcription reaction, the mixture consisted 20 mM rNTPs, 1 × transcription buffer and 1.5 μg PCR products and was incubated at 60 °C for 30 min. Then 100 U T7 RNA polymerase was added to the mixture, followed by incubation at 37 °C for 5 h and 70 °C for 10 min to inactive T7 RNA polymerase. After transcription, single-stranded and double-stranded DNA were digested by 25 U DNase I at 37 °C for 1 h. The products of transcription reaction were characterized by polyacrylamide gel electrophoresis (PAGE) as depicted in Fig. S1. The length of the produced RNA strand was about 100 nt, which was consistent with our design and used as the model target for a substitution of CDR1AS.

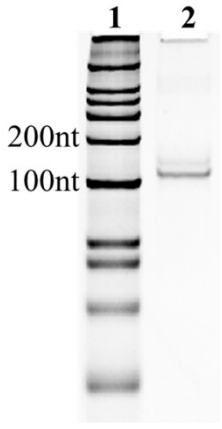

Fig. S1. PAGE analysis of the CDR1AS fragments. Lane 1, RNA ladder marker; lane 2, the CDR1AS fragments.

2. Optimization of the concentration of the four primers in the proposed assay

The amplification efficiency of LAMP reaction mainly depends on the formation of double stem-loop DNA, which is the starting material of LAMP reaction. The formation of double stem-loop DNA relies on the extension of multiple stem-loop primers (SLPs), including the reverse transcription of SLP1, the strand displacement extension of SLP2, SLP3 and P4. Therefore, the effect of the primers on LAMP reaction is investigated. To simplify the experiment, the concentration of the four primers keeps the same in the range of 1 nM to 50 nM. As shown in Fig. S2, only fluorescence curves of 10 fM and 100 fM target can be detected when the concentration of primers is 1 nM, while others remain to be a straight line. This may due to the relatively low efficiency of LAMP reaction as fewer double stem-loop structure DNA formed with lower concentration of four primers. When the concentration of the four primers increases to 10 nM, the fluorescence curves generated by 1 fM to 100 fM target appear in the right order and have the same interval, indicating that as low as 1 fM target can be quantitatively detected. Further increasing the concentration to 50 nM, the signals produced by blank cannot be ignored, indicating that non-specific amplification produced with higher concentration of primers. The POI values produced by each fluorescence curve of different primer concentration are shown in Fig. S2d. Comparing both amplification efficiency and non-specific amplification products, 10 nM primers were selected as the optimal concentration in our assay.

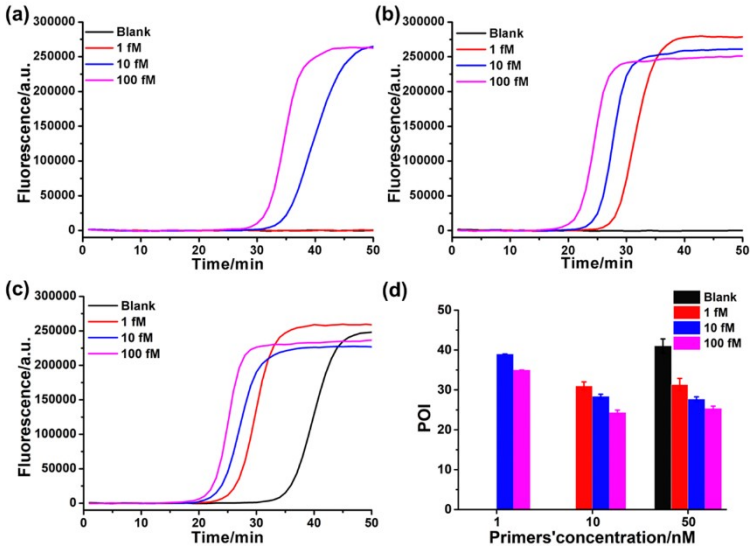

Fig. S2. Real-time fluorescence curves generated by the CDR1AS fragments at concentrations of 100 fM to 1 fM and blank in the presence of different primer concentrations. The concentration of the four primers is (a) 1 nM, (b) 10 nM and (c) 50 nM, respectively. (d) The POI values generated by each fluorescence curve of different primer concentration. Error bar is the standard deviation of triplicate measurements. Other experimental conditions are carried out according to the optimal conditions in the experimental sections.

3. Optimization of the temperature during extension reaction

The formation efficiency of the extension products is affected by the temperature of extension reaction. Taking into consideration of both melting temperature (Tm) of primers and the activity of Bst DNA polymerase, the extension temperature is investigated by detecting CDR1AS fragments at 1 fM to 100 fM with the temperature ranging from 45 °C to 55 °C, respectively. As shown in Fig. S3, when the temperature is 45 °C, the CDR1AS fragments with the concentration of 1 fM are undetected, indicating that the activity of Bst DNA polymerase is too weak to perform the extension reaction at that temperature which has affected the detection sensitivity. When the temperature raised to 50 °C, the fluorescence curve produced by 1 fM target can be well detected and the POI values produced by CDR1AS fragments of different concentrations are in the correct order. Increasing the temperature to 55 °C, the

reaction is delayed and the POI value of 1 fM signal is out of linear range. The POI values produced by each fluorescence curve of different extension temperature are shown in Fig. S3d. In comparison of both amplification efficiency and non-specific amplification products, 50 °C is selected as the optimal temperature in the assay.

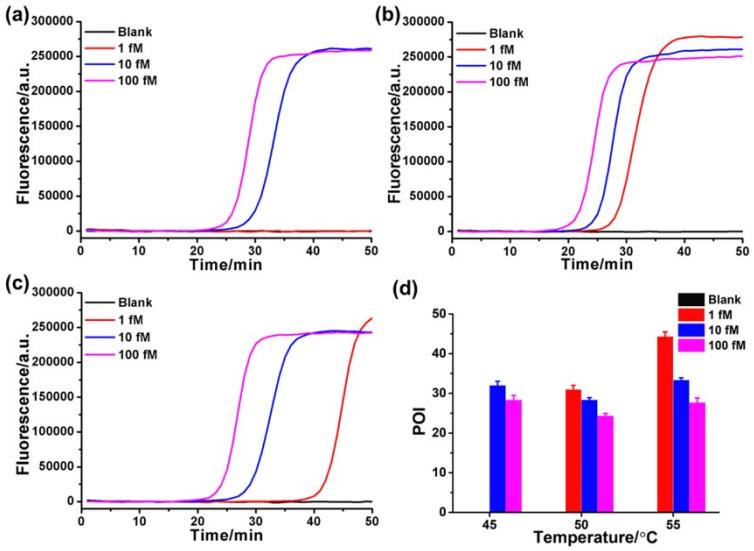

Fig. S3. Real-time fluorescence curves generated at different extension temperature. The temperature is (a) 45 °C, (b) 50 °C and (c) 55 °C, respectively. (d) The POI values generated by each fluorescence curve of different extension temperature. Error bar is the standard deviation of triplicate measurements. Other experimental procedures are the same as described in the experimental section.

4. The influence of Bst DNA polymerase during LAMP reaction

Bst DNA polymerase has relatively high dNTP incorporation efficiency over a wide temperature range while lacking both 5′-3′ exonuclease activity and the ability to perform 3′-5′ proofreading. Due to these properties, it typically induces template-independent exponential amplification, which may start from the formation of primer dimers or hairpins induced by high primer concentration or mismatch events. Because non-specific or undesirable amplifications are able to be further exponentially amplified by this DNA polymerase, the use of Bst DNA polymerase may lead to high rates of false positivity.[1, 2] Therefore, we have optimized the amount of Bst DNA polymerase ranging from 0.8 U to 4 U during LAMP reaction. Lower amount of Bst

DNA polymerase leads to a slower reaction rate. As shown in Fig. S4, when the amount of Bst DNA polymerase is 0.8 U, the efficiency of amplification is relatively low and the fluorescence signal of 1 fM takes about 80 min to reach the plateau, indicating that the amount of Bst DNA polymerase is not enough for LAMP reaction. When the amount of Bst DNA polymerase increases to 2.4 U, all of the real-time fluorescence curves generated from 1 fM to 100 fM target reach plateau within 40 min and the blank curve does not rise. When the amount of the Bst DNA polymerase increases to 4 U, the POI values from 1 fM to 100 fM curves are almost the same, and the blank curve rises around 20 min, indicating the existence of non-specific amplification with excess Bst DNA polymerase. The POI values produced by each fluorescence curve of different amount of Bst DNA polymerase are shown in Fig. S4d. Taking into consideration of both detection sensitivity and specificity, 2.4 U is selected as the optimum amount of Bst DNA polymerase for LAMP reaction.

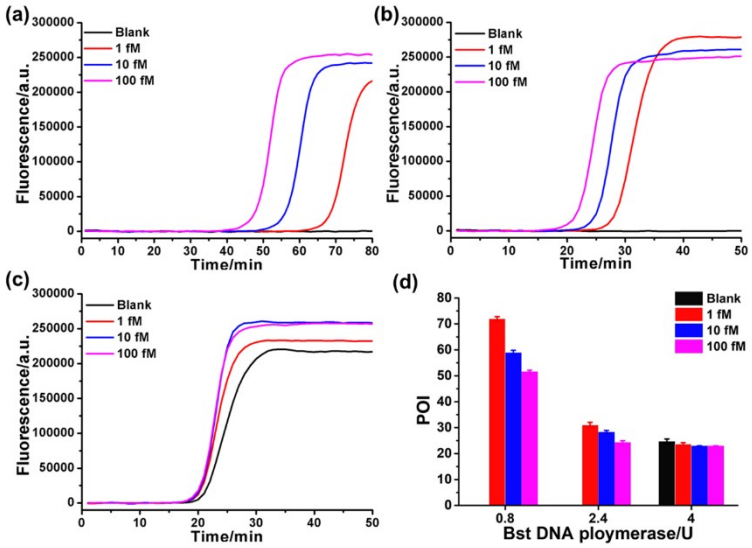

Fig. S4. The influence of Bst DNA polymerase during LAMP reaction. The amount of Bst DNA polymerase is (a) 0.8 U, (b) 2.4 U and (c) 4 U, respectively. (d) The POI values generated by each fluorescence curve of different amount of Bst DNA polymerase. Error bar is the standard deviation of triplicate measurements. Other experimental procedures are the same as described in the experimental section.

5. Verification of the sensitivity using two SLPs induced conventional LAMP assay

In our assay, one circRNA can generate more than two double stem-loop DNAs under the displacement extension of multiple SLPs. With more stem-loop DNAs formed, the sensitivity of our assay is also increased. To figure out the effect of the multiple SLPs, we test the sensitivity of our assay using only two SLPs (SLP1 and SLP2). As shown in Fig. S5, as low as 10 fM circRNA, which is not in the linear range, can be detected. The linear range of the two SLPs induced conventional LAMP assay spans over 3 orders of magnitude from 100 fM to 100 pM. The corresponding linear equation is $POI=-4.29lgC_{CDR1AS}(M)-23.62$ with the correlation coefficient (R^2) of 0.9933. Moreover, it takes about 60 min for 10 fM circRNA to reach plateau, while only 35 min is needed for our multiple SLPs induced cascaded LAMP reaction. These results demonstrate that the sensitivity of multiple SLPs induced cascaded LAMP has increased at least 10-fold compared with that of two SLPs together with accelerated reaction rate.

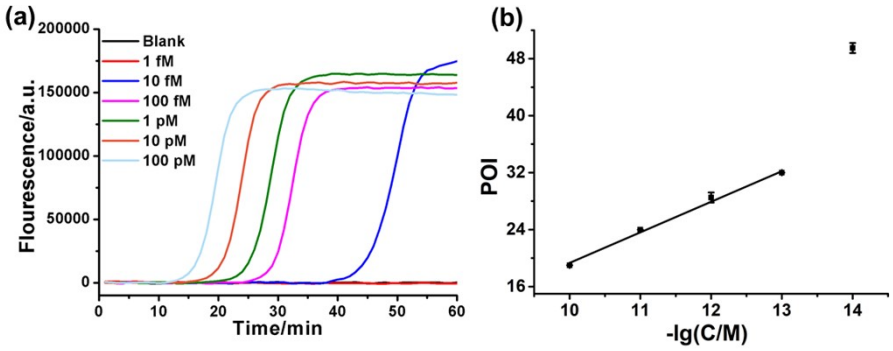

Fig. S5. (a) Real-time fluorescence curves generated by two SLPs induced conventional LAMP assay. (b) The linear relationship between the POI values generated by each fluorescence curve and the logarithm of the concentration of CDR1AS fragments. Error bar is the standard deviation of the data from three experiments. Other experimental procedures are the same as described in the experimental section.

6. PAGE analysis of the cascaded reaction processes of our multiple SLPs induced cascaded LAMP reaction

In order to verify the cascaded reaction processes of our method, we have analyzed the amplification products generated from our multiple SLPs induced cascaded

LAMP reaction and two SLPs induced conventional LAMP reaction. As shown in Fig. S6, both the two reactions generate a wide range of DNA with various lengths which is in accordance with the principle of LAMP reaction. Compared with two SLPs induced conventional LAMP reaction, our multiple SLPs induced cascaded LAMP reaction produces more amplification products and can detect 10 fM circRNA after amplification for 28 min, while two SLPs induced conventional LAMP reaction can barely detect 100 fM circRNA which is the same with real-time fluorescence results as shown in Fig. 3a and S5. These results indicate that the utilization of multiple SLPs indeed accelerates LAMP reaction and improves the detection sensitivity, further verifying the occurrence of cascaded amplification processes.

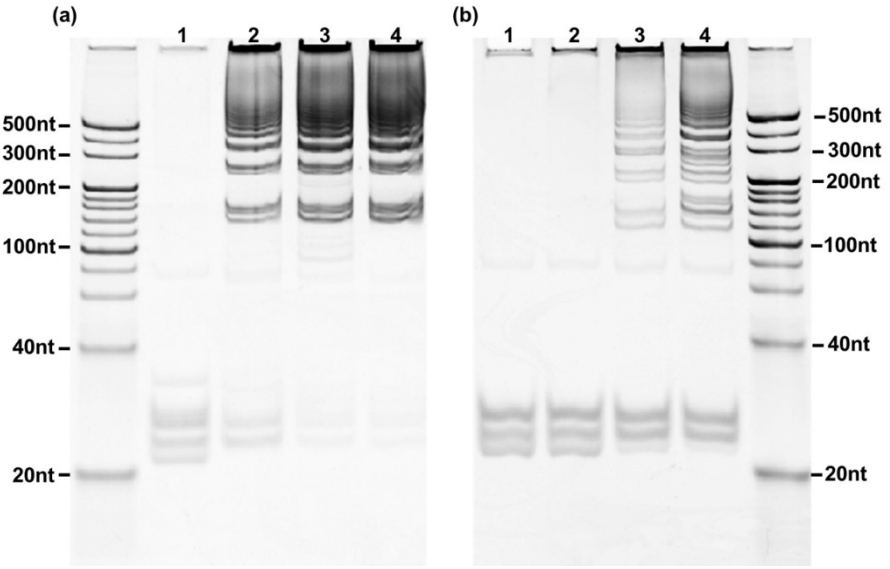

Fig. S6. PAGE analysis of the amplification products generated from (a) our multiple SLPs induced cascaded LAMP reaction and (b) two SLPs induced conventional LAMP reaction at a constant reaction time of 28 min. Lane 1, blank; lane 2, 10 fM circRNA; lane 3, 100 fM circRNA; lane 4, 1 pM circRNA.

7. The influence of miRNA when detecting circRNA using our multiple SLPs induced cascaded LAMP reaction

In order to figure out the influence of miRNA on our multiple SLPs induced cascaded LAMP reaction, we have added a denaturation step by heating to detach miRNAs from total RNA before detecting circRNA. As shown in Fig. S7, the fluorescence

curves generated from total RNA after denaturation are almost the same with those in the absence of denaturation, demonstrating that the presence of miRNA does not affect the detection accuracy of our method.

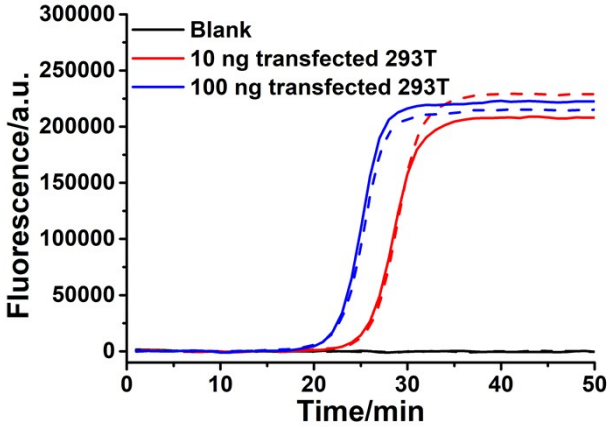

Fig. S7. Real-time fluorescence curves for detecting CDR1AS in total RNA extracted from C1946 transfected 293T cells. The solid lines represent total RNA in the absence of denaturation step and the dotted lines represent total RNA with denaturation step.

8. Verification of the CDR1AS junction site in total RNA extracted from C1946 transfected 293T cells

First, total RNA extracted from C1946 transfected 293T cells was reverse transcribed using random primer and then amplified by PCR using divergent primers as shown in Table 3. Then the PCR products were sequenced by Sangon Biotech Co., Ltd. The sequencing results shown in Fig. S6 indicate that total RNA extracted from C1946 transfected 293T cells has the unique CDR1AS junction site and the specific recognition region between SLP1, SLP2, SLP3, P4 and CDR1AS, demonstrating the existence of circRNA which is in accordance with our results.

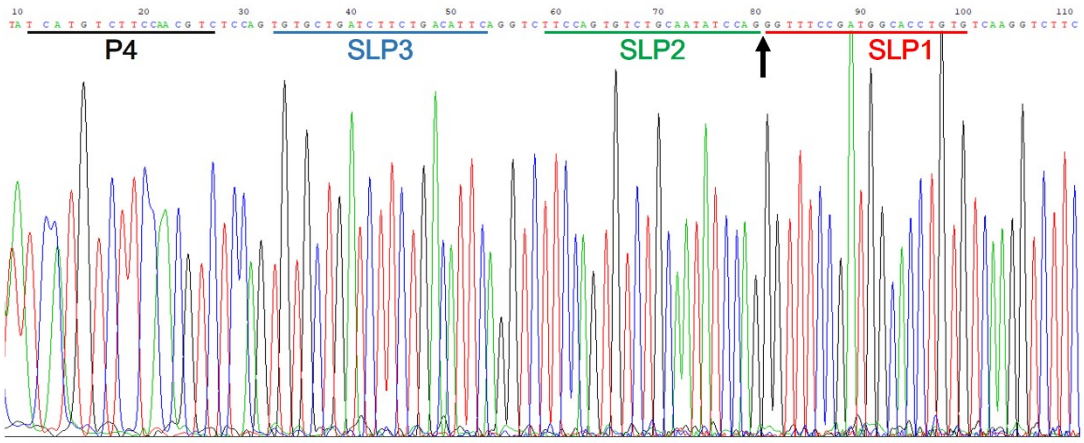

Fig. S8. The sequencing results of CDR1AS in total RNA extracted from C1946 transfected 293T cells. The red line indicates the hybridization sequence between CDR1AS and SLP1. The green, blue and black lines indicate the recognition region between SLP2, SLP3, P4 and CDR1AS, respectively. The black arrow indicates the junction site of CDR1AS.

Table S1. The sequences used for synthesis of CDR1AS fragments.

Name	Sequences (5'-3' direction)
T7 DNA template	*TAATACGACTCACTATAG*GGCTCAATATCCATGTCT TCCAACGTCTCCAGTGTGCTGATCTTCTGACATT CAGGTCTTCCAGTGTCTGCAATATCCAGGGTTTC CGATGGCACCTGTG
Forward primer	*TAATACGACTCACTATAG*GGCTCAATATCC
Reverse primer	CACAGGTGCCATCGGAAAC

Notes: The underlined sequences indicate the complementary sequence between T7 DNA template and primers. The italic sequences indicate the T7 promoter sequence.

Table S2. The sequences used for the detection of circRNA.

Name	Sequences (5'-3' direction)
CDR1AS fragment	rGrGrCrUrCrArArUrArUrCrCrArUrGrUrCrUrUrCrCrAr ArCrGrUrCrUrCrCrArGrUrGrUrGrCrUrGrArUrCrUrUr

	CrUrGrArCrArUrUrCrArGrGrUrCrUrUrCrCrArGrUrGrUrCrUrGrCrArArUrArUrCrCrArG*rGrGrUrUrUrCrCrGrArUrGrGrCrArCrCrUrGrUrG
Linear RNA-1	rGrGrUrUrUrCrCrGrArUrGrGrCrArCrCrUrGrUrGrUrCrArArGrGrUrCrUrUrCrCrArArCrArArCrUrCrCrGrGrGrUrCrUrUrCrC
Linear RNA-2	rGrGrCrUrCrArArUrArUrCrCrArUrGrUrCrUrUrCrCrArArCrGrUrCrUrCrCrArGrUrGrUrGrCrUrGrArUrCrUrUrCrUrGrArArUrArUrCrCrArGrGrUrCrGrUrGrCrArArUrArUrCrCrArGrGrUrCrUrGrCrArArUrArUrCrCrArG
SLP1	CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGATTTTCCTCTGCTGTCGTTTTTCACAGGTGCCATCGGAAACC
SLP2	ATCGTCGTGACTGTTTGTAATAGGACAGAGCCCCGCACTTTCAGTCACGACGATTTTTCCAGTGTCTGCAATATCCAG
SLP3	ATCGTCGTGACTGTTTGTAATAGGACAGAGCCCCGCACTTTCAGTCACGACGATTTTTTGTGCTGATCTTCTGACATTC
P4	TCCATGTCTTCCAACGTC
FIP	CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGA
BIP	ATCGTCGTGACTGTTTGTAATAGGACAGAGCCCCGCAC

Notes: The letter ‘r’ indicates ribonucleotides. The symbol ‘*’ indicates the junction site of circRNA. The underlined sequences indicate the complementary sequences between CDR1AS fragments and SLP1. The dotted underlined sequences indicate same sequences between CDR1AS fragments and SLP2. The bolded underlined sequences indicate same sequences between CDR1AS fragments and SLP3. The

double underlined sequences indicate same sequences between CDR1AS fragments and P4.

Table S3. The sequences of divergent primers used during sequencing.

Name	Sequence (5'-3')
Divergent forward primer	GTCTTCCAACAACTACCCAGTC
Divergent reverse primer	TGGATTTTCTGGAAGCTCAGG

References

1. X. Ye, Y. Li, L. Wang, X. Fang and J. Kong, Chem. Commun., 2018, **54**, 10562-10565.

2. R. R. Garafutdinov, A. R. Gilvanov, O. Y. Kupova and A. R. Sakhabutdinova, Int. J. Biol. Macromol., 2020, **161**, 1447-1455.