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Supplementary Information

A hyperbranched transcription-activated CRISPR-Cas12a signal amplification strategy for sensitive microRNA sensing

Weimin Tian, Xiaoling Liu*, Gaoting Wang and Chenghui Liu*

Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, Key Laboratory of Applied Surface and Colloid Chemistry, Ministry of Education, School of Chemistry and Chemical Engineering, Shaanxi Normal University, 620 West Chang'an Street, Xi'an, Shaanxi, 710119, P. R. China.

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1. Experimental Section

Materials and Apparatus. SplintR ligase and 10× SplintR ligase reaction buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM ATP, 100 mM DTT, pH 7.5), phi29 DNA polymerase and 10× phi29 DNA polymerase reaction buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 40 mM DTT, pH 7.5), T7 RNA polymerase and 10× T7 RNA polymerase reaction buffer (400 mM Tris-HCl, 60 mM $MgCl_2$, 20 mM spermidine, 10 mM DTT, pH 7.9), Cas12a and NEBuffer 2.1 (500 mM NaCl, 100 mM Tris-HCl, 100 mM $MgCl_2$, 1 ng/mL BSA, pH 7.9) were purchased from New England Biolabs (USA). The dNTPs, Ribonuclease inhibitor and RNase-free water were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). NTP mixture was supplied by Sangon (Shanghai, China). All nucleic acids sequences were synthesized and purified by Takara Biotechnology Co. Ltd. (Dalian, China). Sequences of all nucleic acids were listed in Table S1.

Table S1. The sequences of all nucleic acids used in the Cas-TCA.

name	sequence
miR-21	5′-UAGCUUAUCAGACUGAUGUUGA-3′
miR-21-1	5′-UAGCUUAUCAUACUGAUGUUGA-3′
let-7a	5′-UGAGGUAGUAGGUUGUAUAGUU-3′
miR-24	5′-UGGCUCAGUUCAGCAGGAACAG-3′
miR-31	5′-AGGCAAGAUGCUGGCAUAGCU-3′
miR-92a	5′-UAUUGCACUUGUCCCGGCCUGU-3′
miR-205	5′-UCCUUCAUUCCACCGGAGUCUG-3′
miR-195	5′-UAGCAGCACAGAAAUAUUGGC-3′
miR-155	5′-UUAAUGCUAAUCGUGAUAGGGGU-3′
miR-143	5′-UGAGAUGAAGCACUGUAGCUC-3′
padlock probe	5′-phosphate-***CTGATAAGCTA***TAATACGACTCACTATAGGGTA ATTTCTACTGTTGTAGAT***TCAACATCAGT***-3′
21* primer	5′-TCAACATCAGTCTGATAAGCTA-3′
T7* primer	5′-CCCTATAGTGAGTCGTATTA-3′
TS	5′-GCCGGGGTGGTGCCCATCTACCCTATAGTGAGTCGTATT ATAGCTTATCAGACTGATGTTGATAAACGGCCACAAGC-3′
NTS	5′-GCTTGTGGCCGTTTATCAACATCAGTCTGATAAGCTATA ATACGACTCACTATAGGGTAGATGGGCACCACCCCGGC-3′
Fluorophore/quencher-	5′-FAM-TTATT-BHQ1-3′

labeled ssDNA reporter	

Note: In the padlock probe**,** a phosphate group is modified at its 5′-end. The bold italics sequences are hybridization regions for miR-21, the red letters are T7 promoter sequence, and the green letters are the direct repeat sequences of crRNA. The TS and NTS indicate the target strand and non-target strand, respectively. The hybridized TS/NTS serves as the Cas12a/crRNA targeting dsDNA, in which the underline region indicates the PAM sequence. 21* primer is the complementary sequence of miR-21 sequence, T7* primer is complementary to the T7 promoter sequence.

***HRCA reaction*.** First, the ligation reaction was performed in 5 μL mixture containing 1× SplintR ligase buffer, 10 nM padlock probe and an appropriate amount of miR-21 or extracted total small RNAs by adding 2.5 U SplintR ligase. The reaction mixture was incubated at 85 °C for 2 min, 37 °C for 30 min and 95 °C for 10 min. After ligation reaction, 5 μL phi29 DNA polymerase buffer consisted of 250 μM dNTPs, 200 μg/mL BSA, 100 nM 21* primer, 100 nM T7* primer and 5 U phi29 DNA polymerase were added to initiate the HRCA reaction. The HRCA reaction was performed at 30 °C for 2 h and terminated by heating the mixture to 80 °C for 10 min.

***Transcription amplification reaction*.** 2 μL of the HRCA product was transferred to a final 10 μL of transcription reaction mixture containing 0.5 mM NTP, 1× T7 RNA polymerase buffer, 8 U Ribonuclease inhibitor and 5 U T7 RNA polymerase. The transcription amplification was conducted at 37 °C for 3 h and stopped by incubation at 70 °C for 10 min.

***Cas12a-based crRNA processing and trans-cleavage reaction*.** Cas12a-based crRNA processing and cleavage reaction was carried out in 10 μL mixture containing 2 μL of the transcription product, 1× NEBuffer 2.1, 100 nM dsDNA activators, 1 μM fluorophore/quencher-labeled ssDNA reporter and 100 nM Cas12a in 37 °C for 45 min and terminated by incubation at 65 °C for 10 min. After that, the Cas12a cleavage reaction solution was diluted to 100 μL with 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and the fluorescence spectra was record by using an F-7000 fluorescence spectrophotometer (Hitachi, Japan) with the excitation wavelength of 488 nm.

***Gel Electrophoresis*.** The 10% denaturing polyacrylamide gel electrophoresis in 1× TBE buffer was performed to analyse the product of each step. The gel electrophoresis was run at room temperature

with a 110 V constant voltage for 80 min and stained by SYBR Gold for 5 min. Then the gel was visualized on a Gel Doc EZ Imager (Bio-Rad, USA).

Cell culture and total small RNAs extraction. Human breast cancer cell line MCF-7 was obtained from the cell bank of Chinese Academy of Sciences and were cultured in DMEM Medium (GIBCO) which contains 10% (v/v) fetal calf serum, 0.01 mg/ml human recombinant insulin (Sigma), 100 μg/mL streptomycin and 100 U/mL penicillin at 37 °C with 5% CO_2. When the MCF-7 cells grew to 80%, the cells were collected. Then the total small RNAs was extracted according to the RNAiso for Small RNA kit (Takara, China) and quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA).

2. Optimization of the experimental conditions

The concentrations of phi29 DNA polymerase, T7 RNA polymerase and Cas12a have crucial effects on the amplification efficiency. To reach the best assay performance, these parameters were investigated and optimized. In this study, the signal (produced by 300 zmol miR-21) to background (without miR-21) (S/B) ratios were used to evaluate the amplification efficiency. As exhibited in Fig. S1a, the S/B ratio increased quickly until the phi29 DNA polymerase dosage reached 5 U. Further increase of phi29 DNA polymerase dosage will no longer lead to improved S/B ratio. Thus, 5 U phi29 DNA polymerase was selected as the optimal in the Cas-TCA assay.

Besides, as shown in Fig. S1b, the S/B ratio increased gradually when the dosage of T7 RNA polymerase was increased from 1 U to 5 U. While if the amount of T7 RNA polymerase was higher than 5 U, the S/B ratio exhibited a slow decreasing tendency. So, 5 U T7 RNA polymerase was selected in the Cas-TCA assay.

Following the same principle, according to the results shown in Fig. S1c and S1d, 100 nM Cas12a, and 45 min *trans*-cleavage time were adopted as the optimum conditions in the Cas-TCA assay.

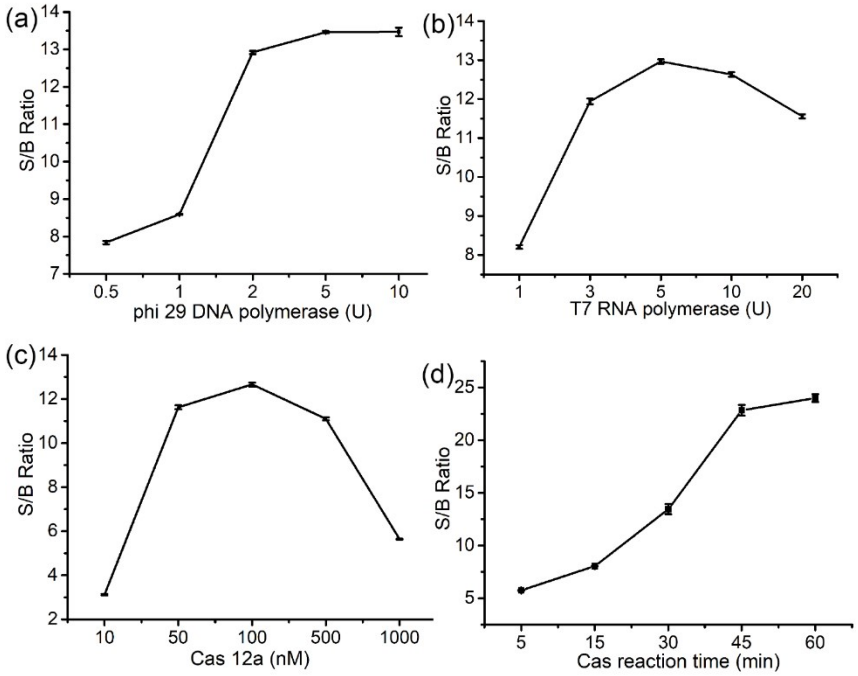

Fig. S1. (a) The effect of the concentration of phi29 DNA polymerase on the S/B ratio of the Cas-TCA for miR-21 analysis. Other condition: T7 RNA polymerase, 5 U; Cas12a, 100 nM; cleavage time of Cas12a, 30 min. (b) The effect of the concentration of T7 RNA polymerase on the S/B ratio of the Cas-TCA for miR-21 analysis. Other condition: phi29 DNA polymerase, 5 U; Cas12a, 100 nM; cleavage time of Cas12a, 30 min. (c) The effect of the amount of Cas12a on the S/B ratio of the Cas-TCA for miR-21 analysis. Other condition: phi29 DNA polymerase, 5 U; T7 RNA polymerase, 5 U; cleavage time of Cas12a, 30 min. (d) The effect of the cleavage time of Cas12a on the S/B ratio of the Cas-TCA for miR-21 analysis. Other condition: phi29 DNA polymerase, 5 U; T7 RNA polymerase, 5 U; Cas12a, 100 nM. The fluorescence intensity (at 520 nm) is produced by 300 zmol miR-21 and background (without miR-21).

3. The comparison of the proposed Cas-TCA with other RCA, T7 transcription, or CRISPR-Cas-based methods for miRNA detection

Table S2. The comparison of the proposed Cas-TCA with other RCA, T7 transcription or CRISPR-Cas-based methods for miRNA detection.

Detection technique	Amplification strategy	Detection limit or the lowest detectable concentration	Reference
Fluorescence	Cascade amplification composed of nicking-mediated RCA and SDA	5 pM	S1
Fluorescence	Target triggered ligation and T7 transcription amplification	3 pM	S2
Fluorescence	RCA-assisted CRISPR/Cas9 cleavage	90 fM	S3
Fluorescence	Amplifying tandem spinach aptamer by polymeric RCA mediated transcription	67.3 fM	S4
Fluorescence	Cas12a self-powered and RCT-unleashed real-time crRNA	0.47 amol	S5
Fluorescence	Target-initiated ligation, transcription and Cas12a cleavage amplification	21.9 fM	S6
Electrochemical method	Catalyzed hairpin assembly and RCA	13.5 fM	S7
Fluorescence	RCA-CEAM dual amplification	12 fM	S8
Fluorescence	Cas-TCA	1 fM (10 zmol in 10 μL)	This work

4. Detection of miR-21 in total small RNAs extracted from MCF-7 cell line

To confirm the accuracy of the Cas-TCA assay, the miR-21 level in the same batch of MCF-7 cell extracts was respectively detected by the proposed Cas-TCA and a stem-loop RT-PCR protocol. The standard procedure of stem-loop RT-PCR method includes two steps: reverse transcription (RT) and real-time PCR. The reverse transcription reaction was carried out in a 6 μL mixture including 1× RT buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 75 mM KCl, pH 8.3), 50 nM stem-loop RT-primer (5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAACATC-3′), 125 μM dNTP, 4 U Ribonuclease inhibitor, 10 U ProtoScrip II reverse transcriptase and an appropriate amount of miR-21 or extracted total small RNAs. The mixture was incubated at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min. Then, the reverse transcription product was transferred to the PCR reaction mixture for real-time quantitative PCR. The PCR mixture including 100 μM dNTP, 200 nM forward primer (5′-ACACTCCAGCTGGGTAGCTTATCAGACT-3′), 0.4× SYBR Green I, 200 nM reverse primer (5′-CTCAACTGGTGTCGTGGAGT-3′), 0.5 U JampStart Taq DNA Polymerase and PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, pH 8.9). The PCR reaction was carried out with a StepOne real-time PCR system (Applied Biosystems, USA) by hot start at 94 °C for 5 min, followed by 50 cycles of 94 °C for 20 s, 60 °C for 1 min and 72 °C for 20 s.

The Fig. S2 shows the comparison results of the two methods, which suggests that Cas-TCA is capable of analyzing miRNA in complex biological samples.

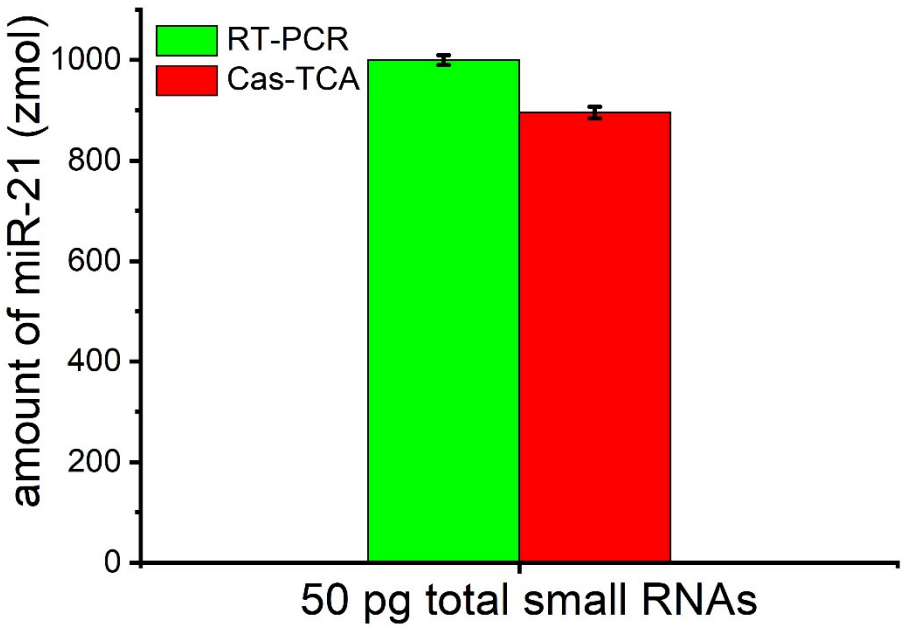

Fig. S2. The comparison of the determined amount of miR-21 in 50 pg total small RNAs extracted from MCF-7 cells by using conventional RT-PCR and our proposed Cas-TCA, respectively.

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