Supporting Information

A sensitive miRNA detection method based on split-T7 switch modulating CRISPR/Cas12a system

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Materials and Methods

Reagents and Materials

The DNA sequence is synthesized and purified by Sangon Biotechnology (Shanghai, China) Co., LTD. The RNA sequence was synthesized and purified by Generay Biotechnology (Shanghai, China) Co., LTD. See Table S1 for details. EnGen Lba Cas12a (Cpf1) was purchased from New England Biolabs. T7 RNA polymerase, NTP purchased from Beyotime Biotechnology (Shanghai, China) Co., LTD. RNase inhibitor was purchased from Takara Bio (Dalian, China). Healthy human serum was obtained from the First Affiliated Hospital of Nanjing Medical University. This study was approved by the Ethics Committee of Nanjing University and Nanjing Medical University. Breast cancer cells (MCF-7) and human embryonic kidney cells (HEK293T) were purchased from the American Type Culture Collection (ATCC). DNA Marker, DEPC-H_2O, TRE reagent, miRNA first strand cDNA synthesis kit (tailing reaction) kit, miRNA fluorescence quantitative PCR kit (SYBR Green Method) were purchased from Sangon Biotechnology (Shanghai, China) Co., LTD. Solution buffer: 10 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.4; 10× transcription buffer: 400 mM Tris-HCl, 20 mM Spermidine, 100 mM $MgCl_2$, 10 mM DTT, pH 7.9; 10× NEBuffer r2.1 buffer: 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 100 μg/mL Recombinant Albumin, pH 7.9.

Polyacrylamide gel electrophoresis (PAGE) analysis

We used 12 % PAGE to characterize the process of three-way junction self-assembly. Each sample was 10 μL in volume and consisted of 100 nM each for S1, S2, S3 and miRNA-21. The samples were placed in a metal bath and incubated at 37 °C for 20 min to fully hybridize the nucleic acids. At the end of the reaction, 2 μL of nucleic acid dye was added to each sample and then electrophoretic analysis was performed at 120 V for 50 min. In addition, we used 8 % PAGE for the characterization of transcription. 8 %

PAGE performed at 100 V for 40 min. The results of electrophoresis were analyzed in an imaging system.

Cell culture

The breast cancer cell line (MCF-7) and human embryonic kidney cell line (HEK293T) used in this work were cultured in a high-sugar DMEM medium (Dulbecco's modified Eagle's medium). The medium was supplemented with 10 % (v/v) fetal bovine serum and 1 % (v/v) penicillin-streptomycin. All cells were cultured in a humidity-controlled incubator at 37 ℃ and 5 % CO_2.

Specific steps for miRNA detection

First, 1 μL sample, 3 μL probe (100 nM each for S1, S2 and S3), 1 μL NTP (10 mM), 2 μL 10× transcription buffer, 0.4 μL T7 RNA polymerase (50 U/μL), 1 μL RNase-inhibitor (40 U/μL) and 11.6 μL DEPC-H_2O mixed with a total volume of 20 μL. Then the reaction was carried out at 37 ℃ for 30 min. After the reaction, it was inactivated at 70 ℃ for 5 min. Then, 2 μL 1 μM Cas12a protein, 2 μL 10 μM FQ fluorescence reporter molecule, 4 μL 500 nM activation DNA (NTS/TS), 5 μL 10× NEBuffer r2.1 buffer, and 17 μL DEPC-H_2O were added to the reaction solution. The total volume is 50 μL. It was mixed and reacted at 37 ℃ for 20 min, then inactivated at 70 ℃ for 5 min. Finally, the reaction solution was transferred into a black 96-well plate, and the fluorescence intensity at 525 nm wavelength (excitation wavelength 485 nm) was measured using a microplate reader (TECAN Infinite 200Pro).

Extraction of total RNA from cell samples

Total RNA was extracted from the cell samples using TRE reagent (Sangon Biology, Shanghai, China). The experimental operation was carried out according to the standard step in the instruction. The steps are as follows. Step 1, 500 μL of the sample is mixed with 1 mL of TRE reagent for cracking, followed by 600 μL of DEPC-H_2O and left for 5 min at room temperature. After centrifugation at 12000 rpm for 15 min, transfer the upper water phase into a clean centrifuge tube. Step 2, add the same volume of isopropyl

alcohol, mix evenly, leave at room temperature for 20 min, centrifuge at 12000 rpm for 10 min, and remove the supernatant. Step 3, add 1 mL 75 % ethanol to clean and precipitate, centrifuge at 12000 rpm for 3 min, and remove the supernatant. Step 4, after drying at room temperature for 3-5 min, DEPC-H_2O was added to dissolve the extracted total RNA, and then stored in an environment of -80 ℃ for subsequent use.

cDNA synthesis and qPCR analysis

The synthesis of cDNA was performed using miRNA first strand cDNA synthesis kit (tailing reaction) (Sangon, Shanghai, China) in accordance with the operation steps of the product instructions. Then the obtained cDNA was analyzed by PCR. qPCR analysis was performed using the miRNA fluorescent quantitative PCR kit (SYBR Green Method) (Sangon, Shanghai, China). The experimental operation was performed according to the standard procedure in the instruction. We used U6 RNA as the internal reference gene for miRNA-21. qPCR reaction procedure: the pre-denaturation process was 95 ℃, 30 seconds; The denaturation process is 95 °C, 5 seconds, then 60 °C, 30 seconds, a total of 40 cycles. Finally, $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression of miR-21 in the samples according to the experimental results.

Table S1. The DNA and RNA sequences used in this work.

Name	Sequence (5' to 3')
Template-1	ACCGA AATAA GATCA GAGAT ATC TAC ACT TAG TAG AAA TTA CCCTATAGTGAGTCGTATTA TTCCCAAACCCGC
Template-2	GCGGGTTTGGGAA TAATACGACTCACTATAGGG
S	ACCGA AATAA GATCA GAGAT ATC TAC ACT TAG TAG AAA TTA CCCTATAGTGAGTCGTATTA TT CTGATAAGCTA
S18	ATACGACTCACTATAGGG
S16	ACGACTCACTATAGGG
S14	GACTCACTATAGGG
S12	CTCACTATAGGG
S2	TCAACATCAGT AA TA
S4	TCAACATCAGT AA TAAT
S6	TCAACATCAGT AA TAATAC
S8	TCAACATCAGT AA TAATACGA
Target	TAGCTTATCAG ACTGATGTTGA
S1-4	ACCGA AATAA GATCA GAGAT ATC TAC ACT TAG TAG AAA TTA CCCTATAGTGAGTCGT ATTA CTGATAAGCTA
S1-5	ACCGA AATAA GATCA GAGAT ATC TAC ACT TAG TAG AAA TTA CCCTATAGTGAGTCGT ATTA T CTGATAAGCTA
S1-6	ACCGA AATAA GATCA GAGAT ATC TAC ACT TAG TAG AAA TTA CCCTATAGTGAGTCGT ATTA TT CTGATAAGCTA
S1-7	ACCGA AATAA GATCA GAGAT ATC TAC ACT TAG TAG AAATTA CCCTATAGTGAGTCGT ATTA TTT CTGATAAGCTA

S3-4	TCAACATCAGT TAAT
S3-5	TCAACATCAGT A TAAT
S3-6	TCAACATCAGT AA TAAT
S3-7	TCAACATCAGT AAA TAAT
miR-21	UAGCUUAUCAG ACUGAUGUUGA
1M	UAGCUUAUAAG ACUGAUGUUGA
2M	UAGCUUAUAAG GCUGAUGUUGA
let-7a	UGAGGUAGUAG GUUGUAUAGUU
miR-17	CAAAGUGCUUACAGUGCAGGUAG
S1-let-7a	ACCGA AATAA GATCA GAGAT ATC TAC ACT TAG TAG AAA TTA CCCTATAGTGAGTCGT ATTA T CTACTACCTCA
S3-let-7a	AACTATACAAC A TAAT
CrRNA	UAA UUU CUA CUA AGU GUA GAU AUCUC UGAUC UUAUU UCGGU
Activation DNA-TS	ACCGA AATAA GATCA GAGAT GAAACT
Activation DNA-NTS	AGTTTC ATCTC TGATC TTATT TCGGT
Fluorescence reporter (FQ)	FAM-TTATTT-BHQ1
miR-21-5p forward primer	TAGCTTATCAGACTGATGTTGA
miR-21-5p reverse primer	TGCGTGTCGTGGAGT
U6 forward primer	GCTTCGGCAGCACATATACTAAAAT
U6 Reverse primer	CGCTTCACGAATTTGCGTGTCAT

Note: The pink sequences represent the transcription template; the purple sequences represent the T7 promoter sequence; the blue sequences represent mutation site.

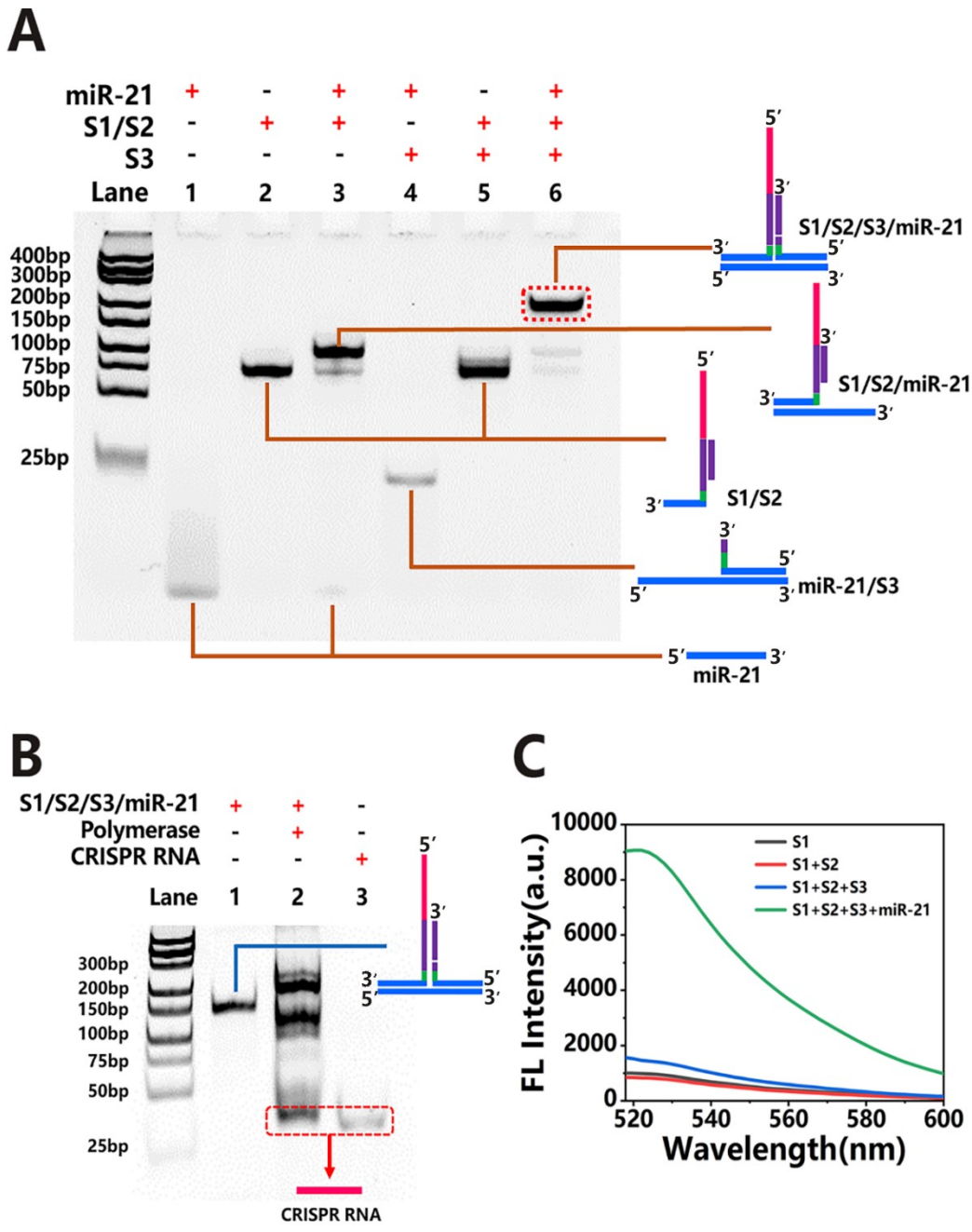

Fig. S1. (A) 12% PAGE analysis was used to verify the three-way junction self-assembly of miR-21 and the detection probe. Lane 1: target miR-21; Lane 2: S1 + S2; Lane 3: miR-21 + S1 + S2; Lane 4: miR-21 + S3; Lane 5: S1 + S2 + S3; Lane 6: miR-21 + S1 + S2 + S3. The concentration of S1, S2, S3 and miR-21 is 100 nM. The reaction was performed at 37 ℃ for 20 min. The electrophoretic analysis was performed at 120 V for 50 min. (B) Electrophoresis analysis of transcripts of the three-way junction

template by 8% PAGE. Lane 1: S1 + S2 + S3 + miR-21; Lane 2: S1 + S2 + S3 + miR-21 + T7 RNA polymerase; Lane 3: Synthetic CRISPR RNA. The concentration of S1, S2, S3 and miR-21 is 100 nM. The concentration of synthetic CRISPR RNA is 50 nM. The transcriptional reaction was performed at 37 ℃ for 30 min, then inactivated at 70 ℃ for 5 min. The electrophoretic analysis was performed at 100 V for 40 min. (C) The corresponding fluorescence spectra of the method under different reaction components. The concentration of S1, S2, S3 is 20 nM. The concentration of miR-21 is 100 pM. The transcriptional reaction is performed at 37 ℃ for 20 min, then 70 ℃ for 5 min. CRISPR reaction time is 10 min. The fluorescence emission wavelength is 525 nm, excitation wavelength is 485 nm.

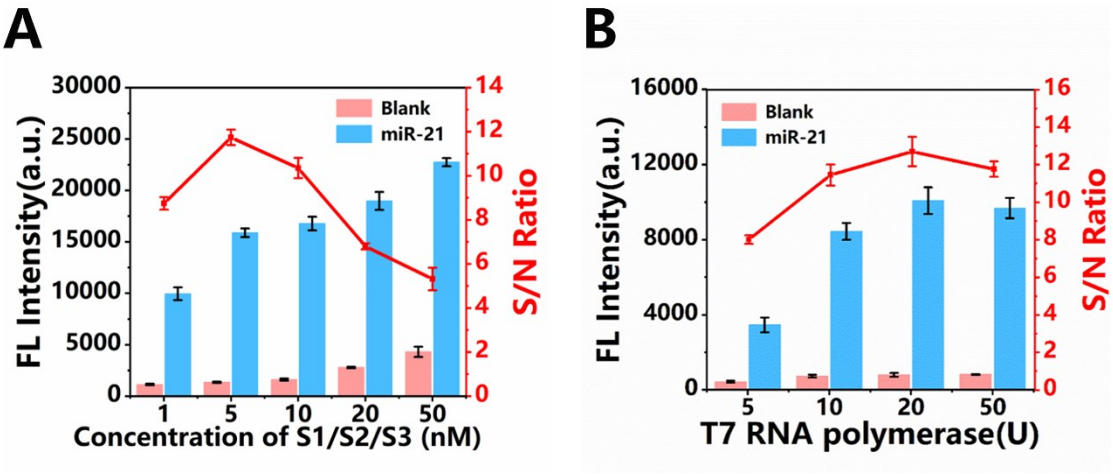

Fig. S2. (A) Optimization of the concentration of the detection probe (S1/S2/S3). The concentration of miR-21 is 100 pM. Transcription is performed at 37 ℃ for 20 min, CRISPR reaction time is 15 min. (B) Optimization of concentration of T7 RNA polymerase used in the transcription. The concentration of S1, S2, S3 is 50 nM, and miR-21 is 100 pM. Error bars: standard deviation (SD), n = 3.

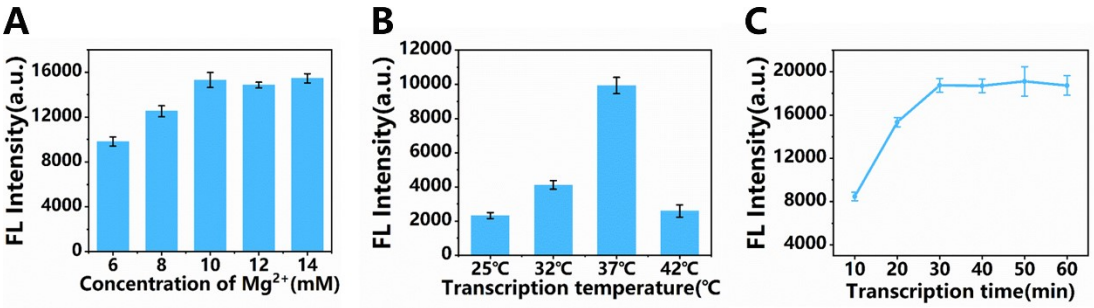

Fig. S3. (A) Optimization of magnesium ion (Mg^{2+}) concentration in transcription reaction (range from 6 mM to 14 mM). (B) Optimization of transcription temperature. (C) Optimization of transcription time. The concentration of S1, S2, S3 is 50 nM, and the concentration of miR-21 is 100 pM. Error bars: standard deviation (SD), n = 3.

Table S2. Comparison of this method with other miRNA detection methods.

Method	LOD	Linear range	Time	References
Colorimetry	2.4 pM	10 pM-100 nM	165 min	[1]
Electrochemistry	79.8 fM	0.1 pM-10 nM	125 min	[2]
Distance	6.28 pM	10 pM-10 nM	130 min	[3]
Fluorescence	161 fM	500 fM-500 pM	160 min	[4]
Fluorescence	43.9 fM	100 fM-1 nM	60 min	This work

Table S3. Recovery tests of the method in ordinary reaction buffer, medium and 10 % human serum.

Sample number	Samples	Added(pM)	Found(pM)	Recovery (%)	RSD (%)
1	Buffer	10	9.94	99.4	3.8
2	Culture medium	10	10.6	106	4
3	10% Serum	10	9.38	93.8	3.9
4	Buffer	100	105	105	2.9
5	Culture medium	100	104.1	104.1	3.2
6	10% Serum	100	96.01	96.01	9.8

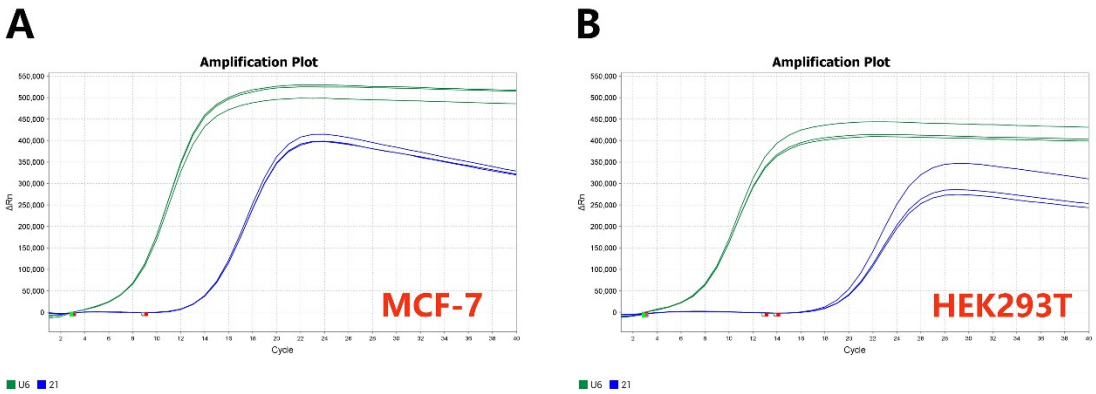

Fig. S4. RT-qPCR curves for the detection of miR-21 and U6 RNA from total RNA extracted from two cell samples. The concentration of total RNA is 2 μg for each sample. Blue: miR-21; Green: U6. U6 is used as the reference gene of miR-21. The average Ct value of miR-21 in breast cancer cell line (MCF-7) was 13.931. The average Ct value of U6 was 7.02. The average Ct value of miR-21 in human embryonic kidney cell line (HEK293T) was 19.529. The average Ct value of U6 was 7.09. The results showed that MCF-7 overexpressed miR-21 compared with HEK293T when U6 RNA expression levels were similar in the above cell lines.

Reference

1. S. H. Gong, J. J. Li, W. Pan, N. Li and B. Tang, *Anal. Chem.,* 2021, **93**, 10719-10726.

2. S. Han, W. Y. Li, S. Yang and R. S. Wang, *ACS Omega,* 2019, **4**, 11025-11031.

3. S. Feng, H. Chen, Z. Hu, T. Wu and Z. Liu, *ACS Appl. Mater. Interfaces,* 2023, **15**, 28933-28940.

4. Z. Lu, W. Ni, N. Liu, D. Jin, T. X. Li, K. Li, Y. L. Zhang, Q. F. Yao and G. J. Zhang, *Microchem. J.,* 2023, **187**, 108370.