Rolling Circle Amplification-Based DNAzyme Walker against Intracellular Degradation for Imaging Tumor Cell microRNA

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1. Section A: Experimental Procedures

SA.1 Materials and reagents

All DNA oligonucleotides (listed in **Table S1**) were synthesized by Shanghai Sangon Biological Engineering Technology Service Co., Ltd. (Shanghai, China). T4 DNA ligase (400 U/μL), deoxynucleotide solution mixture (dNTPs, 10 μM) and phi29 polymerase (10 U/μL) were ordered from Vazyme Biotech Co., Ltd. (Jiangsu, China). HeLa cells (human cervical carcinoma cell line) and MCF-7 cells (human breast cancer cell line) provided by Pricella (Wuhan, China).

Exonuclease I (Exo I, 5 U/μL) and Exonuclease III (Exo III, 100 U/μL) were purchased from Novoprotein Scientific Inc. (Shanghai, China). Glycogen obtained from Shanghai YuanYe Bio-Technology Co., Ltd. (Shanghai, China), Loading buffer(6× and 2×), Dulbecco's Modified Eagle Medium (DMEM) and PBS (pH=7.2~7.4) were purchased from Beijing Solarbio Science & Technology Co.,Ltd. (Beijing, China), DNase I and 17β-Estradiol (E_2) were bought from Beyotime Biotechnology Co., Ltd (Shanghai, China), Hoechst 33342 , DNA Marker and SYBR Green I obtained from BioSharp (Beijing, China), penicillin-streptomycin (PS) and fetal bovine serum (FBS) purchased from Gibco (USA).

SA.2 Construction of the RCA-D-Walker

Preparation of RCA product (RCA-p)

First, the primer (0.5 μM), the template (0.5 μM), T4 DNA ligase buffer, and ultrapure water were mixed to construct the circular template. The resulting solution (19 μL) was annealed at 90 °C for 5 min to facilitate annealing and then slowly cooled to room temperature to produce circular structures. Subsequently, 1 μL of T4 DNA ligase was added, followed by incubation at 16 °C for 8 h. The enzyme was then inactivated by heating at 65 °C for 10 min. Finally, 20 μL of the resulting circular DNA template, 4 μL of 10× phi29 DNA polymerase buffer, 1 μL of dNTPs and 0.5 μL of phi29 DNA polymerase were mixed to obtain the RCA solution. After adjusting the volume to 40 μL with ddH_2O, the mixture was incubated at 30 °C for 30 min. The RCA

reaction was terminated by inactivating phi29 DNA polymerase at 65 °C for 10 min, then the RCA product (RCA-p) was obtained after purification.

Purification of the RCA-p

Added an equal volume of phenol, chloroform, and isopentanol mixed solution (volume ratio 25:24:1) to the long-chain products generated from the RCA reaction, vortexed for 20 s, and then centrifuged at 12000 rpm for 5 min at room temperature. Transferred the supernatant and added 1/10 volume of 3 M sodium acetate, 2.5 volumes of absolute ethanol, and 5 μL of DNA precipitation reagent. Following mix and then frozen at -80°C for 2 h. Subsequently, it was centrifuged at 16000 rpm for 30 min at 4°C. Finally, the mixture was washed twice with 75% ethanol and dried with air, then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to obtain RCA-p.

Preparation of D-MB

The mixed solution of Lock-d (2 μM), DNAzyme (2 μM) and TAMg buffer (40 mM Tris, 7.6 mM $MgCl_2 \cdot 6H_2O$, pH 7.40) was annealed at 90 °C for 5 min and then cooled to room temperature to obtain 10 μL of Locked-DNAzyme. The molecular beacon (MB) was prepared using F-S (2 μM), D-S (2 μM) and TAMg buffer in the same amounts and following the same procedure as described above. Eventually, for **Figure 1** and **Figure S3**, the D-MB solution (20 μL, 1 μM) was obtained by mixing the two components at room temperature for 2 h.

For other experiments, the pre-prepared D-MB and MB were mixed at a 1:4 molar ratio before proceeding with further assembly.

Preparation of RCA-D-Walker

The pre-prepared D-MB solution (1 μM), 1 μL of RCA solution, and 2 μL of Linker were mixed together. After adjusting the volume to 40 μL with TAMg, the mixture was incubated at room temperature for 2 h to obtain the RCA-D-Walker solution (40 μL, 0.5 μM). The concentration of F-S was used to define the concentration of RCA-D-Walker.

SA.3 Gel electrophoretic analysis

For the 15% native polyacrylamide gel electrophoresis (nPAGE) analysis, 8 μL of the corresponding DNA sample solution, 2 μL of 6× loading buffer, and 2 μL of SYBR

Green I were mixed and loaded into the wells. For the 10% denatured polyacrylamide gel electrophoresis (dPAGE) analysis, 5 μL of the DNA samples were mixed with 5 μL of 2× loading buffer, heated at 95°C for 7 min, and then cooled on ice for 2 min before loading into the appropriate wells. Both gel analyses were performed at a constant voltage of 80 V in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA). The gel images were visualized on chemiDox XRS Imaging system (Bio-RAD, U.S.A.).

The sample preparation method for Figure 1A is as follows:

For lane 1-2: the corresponding DNA sequence (0.5 μM) was added to TAMg buffer to total volume of 20 μL each. After thoroughly mixing, the solution was annealed at 90°C for 5 min and then gradually cooled to room temperature.

For lane 3: Template and Primer strand (0.5 μM each) were mixed adjusting the volume to 20 μL with TAMg, and annealed at 90 ℃ for 5 min. After cooling gradually down to room temperature.

For lane 4: The same sample was prepared according to the procedure adopted in lane 3, but T4 DNA ligase buffer instead of TAMg to adjust the total volume to 19 μL, followed by the addition of 1 μL of T4 DNA ligase and incubation at 16 °C for 8 h. Finally, heating at 65 °C for 10 min.

For lane 5: The same sample was prepared according to the procedure adopted in lane 4. Subsequently, 4 μL of 10× Exo I and Exo III buffer, 0.5 μL of Exo I and Exo III, and 11 μL of ddH_2O were mixed and incubated at 37 °C for 1 h, followed by heating to 80 °C for 20 min.

For lane 6: The same sample was prepared according to the procedure adopted in lane 4. Subsequently, 4 μL of 10× phi29 DNA polymerase buffer, 1 μL of dNTPs, 0.5 μL of phi29 DNA polymerase and 14.5 μL of ddH_2O were mixed and incubated at 30 °C for 30 min, followed by heating to 65 °C for 10 min.

The preparation method of samples for Figure 1C was follows:

RCA-p was prepared according to the section of **SA.2**.

For lane 1-2: the corresponding DNA sequence (0.5 μM) was added to TAMg buffer to total volume of 20 μL each. After thoroughly mixing, the solution was annealed at

90°C for 5 min and then gradually cooled to room temperature.

For lane 3: The RCA-p (0.5 μM) and Linker (1 μM) were mixed together adjusting the volume to 20 μL with TAMg, and annealed at 90 ℃ for 5 min. After cooling gradually down to room temperature.

For lane 4: The D-MB prepared as described in the "**Preparation of D-MB**" (20 μL, 1 μM).

For lane 5: The same sample was prepared according to the procedure adopted in lane 1-2 and lane 4, taking 1 μL, 2 μL, and 17 μL, respectively. After thoroughly mixing, the solution was annealed at 90°C for 5 min and then gradually cooled to room temperature.

The preparation method of samples for Figure 1D was follows:

RCA-D-Walker was prepared according to the section of **SA.2**.

For lane 1: The pre-prepared RCA-D-Walker was mixed with 6.5 μL of ddH_2O to obtain the sample (0.5 μM).

For lane 2: The pre-prepared RCA-D-Walker and 2.5 μL of D-miR-21 were mixed thoroughly, then adjusting the volume to 40 μL with ddH_2O, the resulting solution (0.5 μM) was incubated at room temperature for 10 min.

For lane 3: The pre-prepared RCA-D-Walker and 4 μL of NaCl (5 M) were mixed thoroughly, then adjusting the volume to 40 μL with ddH_2O, the resulting solution (0.5 μM) was incubated at room temperature for 10 min.

For lane 4: The pre-prepared RCA-D-Walker, 2.5 μL of D-miR-21 and 4 μL of NaCl (5 M) were mixed thoroughly. The resulting solution was incubated at room temperature for 10 min.

For gel electrophoretic analysis of Figure 1D, no additional DNA fluorescent dyes were added to the samples, and the brightness of the bands originated solely from FAM.

SA.4 AFM imaging

The purified RCA-p were characterized with AFM (a MultiMode 8 atomic force microscope, Bruker, Germany). For AFM imaging, 8 μL of the diluted samples (5-fold dilution) was dropped onto the mica for 20 min. Subsequently, the mica was washed

several times with ultra-pure water to remove unabsorbed samples and dried with N_2. Then, scanning was performed in ScanAsyst mode (Cantilever Type: ScanAsyst-Air).

SA.5 Anti-degradation assay

To study the stability of RCA-D-Walker, D-MB and RCA-D-Walker (35 μL，0.5 μM) were prepared according to the section of **SA.2**, but short chains instead of D-S. 4 μL of the samples were incubated with an equal volume of DNase I (2 U/mL) at 37° C for different time intervals (0, 30, 60, 90, and 120 min). After mixing with EDTA (0.9 μL, 25 mM), the samples were incubated at 65°C for 10 min before undergoing dPAGE analysis.

SA.6 Fluorescence detection

To validate the responsiveness of RCA-D-Walker to the target, RCA-D-Walker (40 μL，0.5 μM) and NaCl (10 μL, 5 M) were mixed adjusting the volume to 200 μL with TAMg, after introducing the D-miR-21 (1 μL, 10 μM), fluorescence detection was performed immediately at room temperature for 15 min. For the blank group, 1 μL of ddH_2O was used instead of D-miR-21, and D-MB was used instead of RCA-D-Walker, while the other steps remained the same as described above.

To investigate the ability of RCA-D-Walker to quantitatively analysis the target, RCA-D-Walker (40 μL, 0.5 μM) and NaCl (10 μL, 5 M) were mixed and diluted to 200 μL with TAMg at different concentrations of D-miR-21 (1 μL each). Fluorescence was measured after 4 h incubation at 37°C. For the blank group, 1 μL of ddH_2O was used instead of D-miR-21, and the experimental methods were same.

For Figure 2C and 2D**,** the detection method was consistent with the above, except that the D-miR-21 was replaced by mutant targets or DNA analogs of non-target miRNAs, and the final concentration was 50 nM. The relative fluorescence intensity was calculated using the formula $(F-F_0)/(F_t-F_0)\times100\%$, with the relative fluorescence intensity of D-miR-21 set as 100%. F denotes the fluorescence intensity induced by mutant targets or DNA analogs of non-target miRNAs, while F_t and F_0 denote the fluorescence intensity induced by the target D-miR-21 and the blank, respectively.

Error bars represent the standard deviations obtained from three parallel assays.

Fluorescence detection was conducted on the F-4600 fluorescence spectrophotometer (Hitachi, Japan) with an excitation wavelength of 488 nm, an emission wavelength of 520 nm, both slits set to 5 nm, and a voltage of 800 V.

SA.7 Statistical analysis

For Figure 2 (C) and 2(D), the t-test was used to determine the significance difference between the control and experimental groups, with ****$p<0.0001$. For Figure 4 (C), the t-test was used to determine the significance difference between the control and experimental groups, **$p<0.01$.

SA.8 Confocal fluorescence imaging

To evaluate the imaging capability of RCA-D-Walker on miRNA-21 in tumor cells, HeLa cells were used in confocal fluorescence imaging experiments. HeLa cells were cultured in complete DMEM medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin) at 37°C in a humid atmosphere with 5% CO_2 for 24 h until they reached 80% confluence. Following several washes with 500 μL of PBS, HeLa cells were incubated with freshly prepared mixture containing RCA-D-Walker (50 μL, 0.5 μM) and 450 μL of DMEM in a humidified incubator at 37°C with 5% CO_2 for 4 h. Following wash with PBS to remove the RCA-D-Walker, the cells were washed three times with 500 μL PBS and fixed with 4% paraformaldehyde for 15 min. The cells were washed three additional times with 500 μL of PBS and subsequently stained with 500 μL of Hoechst 33342 (10 $\mu g \cdot mL^{-1}$) at 37°C for 15 min to label the nuclei. Prior to confocal fluorescence imaging, the cells were washed again with 500 μL of PBS to eliminate the staining solution. The D-MB group and RCA-R-Walker group served as control groups, where D-MB and RCA-R-Walker (We named the RCA product obtained by Random instead of Template as RCA-p-R, assembled with D-MB) replaced RCA-D-Walker. All other experimental steps were the same as the RCA-D-Walker group.

To evaluate the ability of RCA-D-Walker to distinguish between different expression levels of miRNA-21 in cells, we performed confocal fluorescence imaging experiments

using MCF-7 cells. MCF-7 cells were treated with E_2 (17β-Estradiol) to reduce the expression level of miRNA-21. Specifically, E_2 solution (0.5 μL, 10 μM) was added to 500 μL of complete DMEM medium, the mixture was used for culturing MCF-7 cells for 24 h until they reached 80% confluence. The Cy5-labelled RCA-D-Walker assemble according **SA.2** except the Linker was instead of Linker-Cy5. The prepared cells (E_2-treated or untreated MCF-7) were washed three times with 500 μL PBS and then incubated with mixture containing Cy5-labelled RCA-D-Walker (50 μL, 0.5 μM) and 450 μL of DMEM for 4 h, and the other steps are the same as the above described.

Confocal fluorescence imaging was performed by A1+ CellManipulator Plus confocal microscope (Nikon, Japan). The Hoechst, FAM and Cy5 were set to 405 nm, 488 nm and 638 nm laser excitation, respectively. Used NIS-Elements Viewer 5.21 to analyze the confocal fluorescence imaging.

SA.9 Flow cytometry analysis

HeLa cells were seeded into a 6-well plate and culture for 24 h. The RCA-D-Walker, RCA-R-Walker and D-MB (100 μL, 0.5 μM each) diluted in 1 mL DMEM medium incubator 4 h respectively. Subsequently, the cells were digested using trypsin and washed with 500 μL of PBS until the suspension becomes colorless. Finally, resuspend in 300 μL of PBS and obtain a suspension through a 300-mesh nylon filter for flow cytometry detection (Cutoflex, Beckman Coulter).

SA.10 Quantitative reverse transcription-PCR (qRT-PCR) analysis

Total RNA was extracted from MCF-7 cells pre- and post-E_2 treatment using the RNA isolator Total RNA Extraction Reagent Kit. The cDNA was obtained through the miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) method. The resulting cDNA was used as a template for qPCR analysis with the miRNA Universal SYBR qPCR Master Mix kit on the Quant Studio 1 plus qPCR instrument (Thermo Fisher, USA) The amplification process is as follows: denaturation at 94 °C for 5 min followed there were 40 amplification cycles comprising 95 °C for 10 s, 60 °C for 60 s, then 95°C for 15 s finally. U6 was chosen as the internal control, and the relative miRNA-21 expression amount were analyzed using the $2^{-\Delta\Delta Ct}$ method. The reagent kits used for

qRT-PCR were all purchased from Vazyme Biotech Co., Ltd (Nanjing, China).

2. Section B: Supporting Tables

Table S1. DNA sequences designed in the work[a]

	Names	Sequences(from 5'to 3')
RCA	Primer	GAACAGAGAAGGGATATGATAGTGAGTCGTATTAACG TACCAACAA
	Template	**P-** CATATCCCTTCTCTGTTCTCTAACCGTACAGTATTTTCC CGGCGGCGCAGCAGTTAGATTTGTTGGTACGTTAATAC GACTCACTAT
	RCA product	ATAGTGAGTCGTATTAACGTACCAACAAATCTAACTG CTGCGCCGCCGGGAAAATACTGTACGGTTAGAGAACA GAGAAGGGATATG
DNAzyme based-molecular beacon (D-MB)	DNAzyme	GCGGCGGTACCAGGTCAAAGGTGGGTGAGGGGACGC CAAGAGTCCCCGCGGTTAGATAGAGATTAGCTTATCA GACT
	Lock-d	TCAACATCAGTCTGATAAGCTAATCTCTAT
	FAM-Substrate (F-S)	**FAM-** CTGAGCAGTTTTTTTTCTACTCTATCTAT/**rA**/GGAAGTA CC GCCGCTGCTTTTTTCTGCTCAGTACTCTCTTAACTCTC
	Dabcyl-Short strand (D-S)	GACATCTGTCTTTTTTTTGAGAGTTAAGAGAGTA-**Dabcyl**
Other related-DNA strands	Linker	AAAAAAAGACAGATGTCTTTTTGTTGGTACGTTAATAC GACTCACTAT
	Random	**P-** CATATCCCTTCTCTGTTCTGGTTTATATCAGAGTGGAA GGCAGTTGACAACATACTCGCTTGTTGGTACGTTAATA CGACTCACTAT
	Cy5-Linker	AAAAAAAGACAGATGTCTTTTTGTTGGTACGTTAATAC GACTCACTAT-**Cy5**
	Substrate	CTGAGCAGTTTTTTTTCTACTCTATCTAT/**rA**/GGAAGTA CC GCCGCTGCTTTTTTCTGCTCAGTACTCTCTTAACTCTC
	Short strand	GACATCTGTCTTTTTTTTGAGAGTTAAGAGAGTA
Lock strands of other lengths	Lock-a	TCAACATCAGTCTGATAAGCTAATCTCTATCTA
	Lock-b	TCAACATCAGTCTGATAAGCTAATCTCTATCT
	Lock-c	TCAACATCAGTCTGATAAGCTAATCTCTATC
	Lock-e	TCAACATCAGTCTGATAAGCTAATCTCT
	Lock-f	TCAACATCAGTCTGATAAGCTAATCTC
Mismatched non-targets	mismatch-1 (mis-1)	TATCTTATCAGACTGATGTTGA
	mismatch-2 (mis-2)	TATATTATCAGACTGATGTTGA

	mismatch-3 (mis-3)	TATATTATAAGACTGATGTTGA
	mismatch-4 (mis-4)	TATATTATAATACTGATGTTGA
	mismatch-5 (mis-5)	TATATTATTATAATGATGTTGA
Target or non-targets	D-miR-21	TAGCTTATCAGACTGATGTTGA
	D-miR-141	TAACACTGTCTGGTAAAGATGG
	D-let-7d	AGAGGTAGTAGGTTGCATAGTT
	D-miR-200b	TAATACTGCCTGGTAATGATGA
	D-miR-429	TAATACTGTCTGGTAAAACCGT
Primers for Quantitative Real-time PCR	21-Stem-loop primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACTCAACA
	F-primer-miR-21	GCGCGTAGCTTATCAGACTGA
	R-primer-miR-21	AGTGCAGGGTCCGAGGTATT
	6-Stem-loop primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACAACGCT
	F-primer-U6	GCGCGCTCGCTTCGGCAGCACA
	R-primer-U6	AGTGCAGGGTCCGAGGTATT

[a]The base sequences in the underlined parts of Primer and Template are complementary. The base sequences of yellow background in DNAzyme and Lock strands (Lock-a, Lock-b, Lock-c, Lock-d, Lock-e, Lock-f) were complementary; the base sequences of the green font in DNAzyme and F-S complement each other; the two red font base sequences of F-S complement each other; the blue font base sequences in F-S and D-S are complementary. The purple font base sequences of Linker and D-S complement each other; the blue background base sequences in RCA product and Linker are complementary; the two green background base sequences of RCA product complement each other; the underlined base sequences in Lock strands and D-miR-21 are complementary; the red background parts in Mismatched non-targets represent mismatched bases.

Table S2. The average Ct values of miR-21 in pre- and post-E_2 treatment MCF-7 cells determined by qRT-PCR[b]

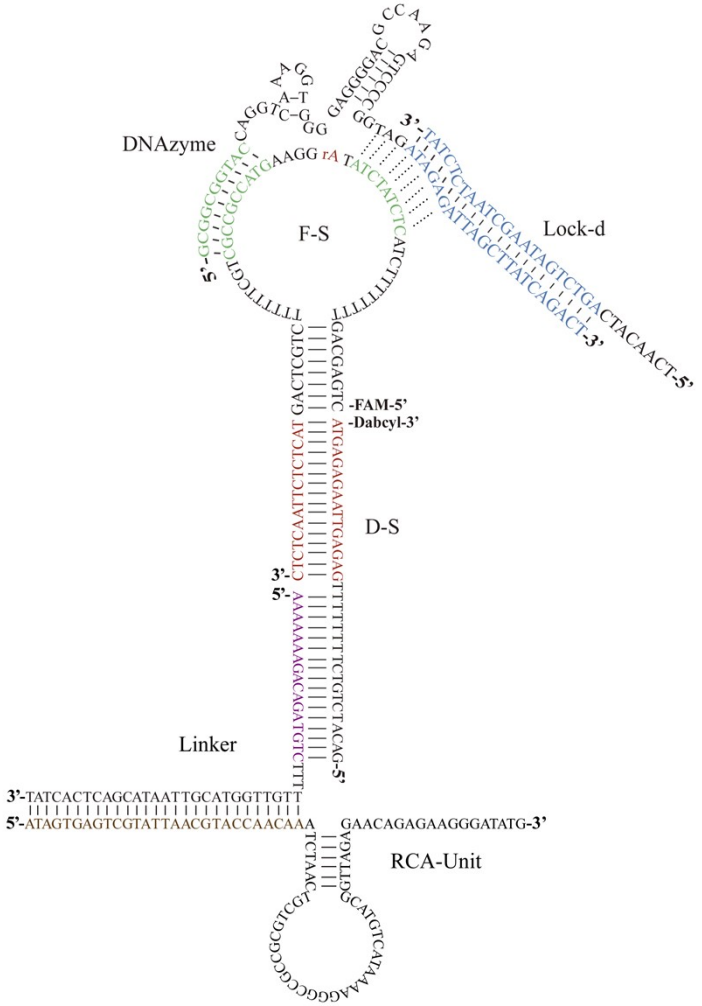
Cell line	miR-21	U6	ΔCt	ΔΔCt	$2^{-(\Delta\Delta Ct)}$
MCF-7	21.515	21.578	0.063	0.000	1.000
MCF-7-E_2	21.564	22.847	1.283	1.220	0.429

[b]The relative miRNA-21 expression amount under the influence of E_2 was calculated by using the $2^{-\triangle\triangle Ct}$ method. The corresponding amplification curves were presented in Figure S6.

Table S3. Comparison of imaging performance of RCA-D-Walker in live cells and literatue methods.

Number	Method	LOD	The basis of cell penetration	Biostability	Ref
1	Genetically encoded fluorescent RNA sensor	0.3 nM	Lipofectamine 3000	/	[1]
2	DNAzyme mediated signal amplification	23 pM	Lipofect-amine 3000	At least 50 min in DNase I (50 U/mL).	[2]
3	Ratiometric fluorescent biosensor	0.2 nM	Protonated Phenyl-doped carbon nitride nanosheets	/	[3]
4	MnO_2 nanotube-based probes	0.6 nM	MnO_2 nanotube	/	[4]
5	Hairpin-fuelled catalytic beacons	67 pM	Gold nanoparticles	At least 60 min in DNase I.	[5]
6	DNAzyme-mediated signal amplification	93 pM	DNA nanostructure based on RCA products	At least 120 min in DNase I (1 U/mL) and 240 min in cell lysate.	This study

3. Section C: Supporting Figures



Scheme S1. Base-paired oligonucleotide sequence assembly of RCA-D-Walker. These sequences marked by the black dots between DNAzyme's right arm and F-S can be hybridized when the complementary region (blue) of Lock-d and DNAzyme is opened, that is, after the target is competitively hybridized with Lock-d.

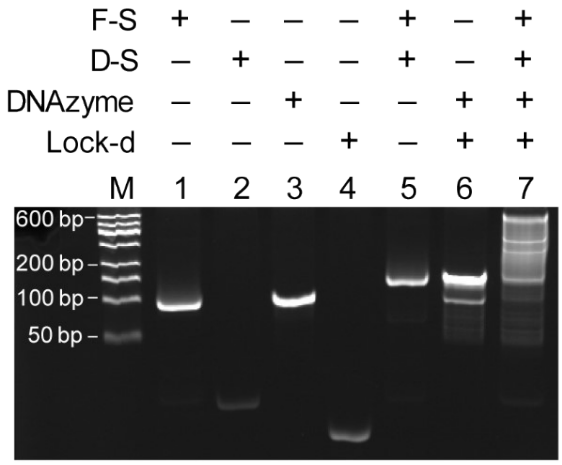


Figure S1. 15% nPAGE analysis of D-MB assembly. Lane 1, F-S; lane 2, D-S; lane 3, DNAzyme; lane 4, lock-d; lane 5, F-S +D-S; lane 6, DNAzyme + lock-d; lane 7, F-S + D-S + DNAzyme + lock-d.

Experimental procedure:

The experimental procedure was consistent with the "**SA.3 Gel electrophoretic analysis**" section. The samples were prepared as follows: The corresponding DNA sequences were diluted with TAMg to a total volume of 10 μl, then heated at 90°C for 5 min and gradually cooled to room temperature.

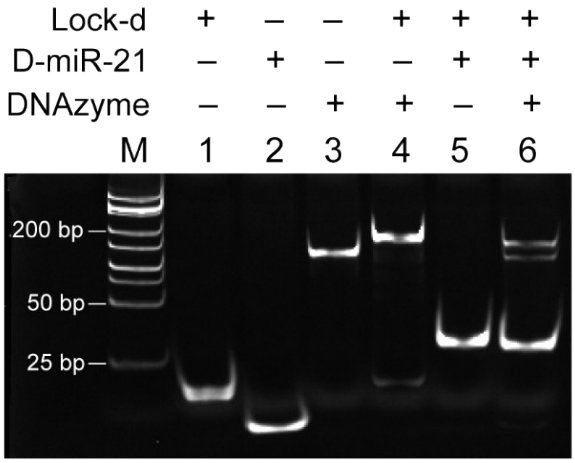


Figure S2. 15% nPAGE analysis of the response of Locked-DNAzyme to the target D-miR-21.

Experimental procedure:

For lanes 1-3: The corresponding DNA sequence (1 μM each) were diluted with TAMg to a total volume of 10 μL, then heated at 90°C for 5 min and gradually cooled to room temperature.

For lane 4 and lane 5: Two corresponding DNA strands (1 μM each) were diluted in TAMg to total volume of 10 μL each. After thoroughly mixing, the solution was annealed at 90°C for 5 min and then gradually cooled to room temperature.

For lane 6: 1 μL of DNAzyme, 1.2 μL of lock-d and 6.3 μL of TAMg were mixed, and the solution was annealed at 90°C for 5 min then gradually cooled to room temperature. Subsequently, the mixture was co-incubated with 1.5 μL of D-miR-21 at room temperature for 1 h.

Discussion:

Lanes 1-3 respectively represent the lock-d, D-miR-21 and DNAzyme band. In lane 4, a new band is clearly visible, indicating the generation of the locked-DNAzyme strand hybrid. Additionally, a faint band is present that migrates at the same rate as the lock-d band, suggesting that this band corresponds to the remaining unreacted lock-d. In lane 5, both the target D-miR-21 band and the lock-d strand band disappeared, being replaced by a new band. This indicated the formation of the target D-miR-21/lock-d strand hybrid. In lane 6, a band with similar brightness and position to lane 5 could be observed. This indicated that the lock-d strand readily hybridized with the target D-miR-21, thereby activating the previously inactive DNAzyme.

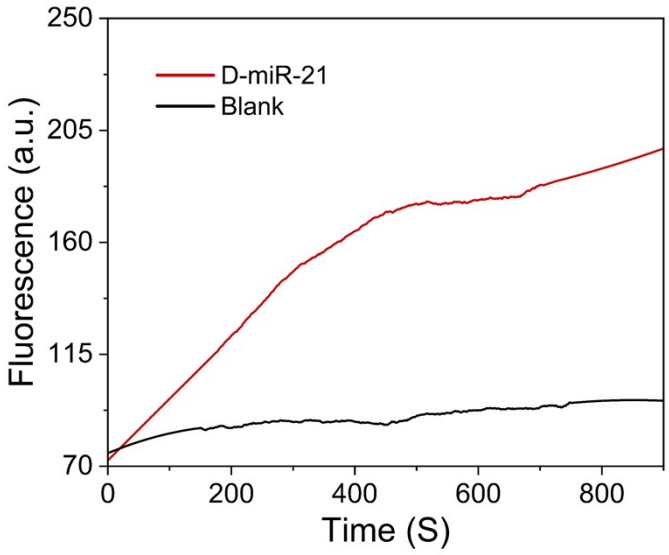


Figure S3. Real-time fluorescence intensity monitoring of D-MB in response to D-miR-21.

Discussion:

This figure illustrates the real-time fluorescence changes of D-MB in response to D-miR-21. In the absence of D-miR-21, the black curve remains essentially level at which point it can be assumed that no fluorescence signal is generated. When D-miR-21 is present, the fluorescence intensity of D-MB begins to rise and then reaches a steady level (red curve), demonstrating that D-MB can respond rapidly to D-miR-21.

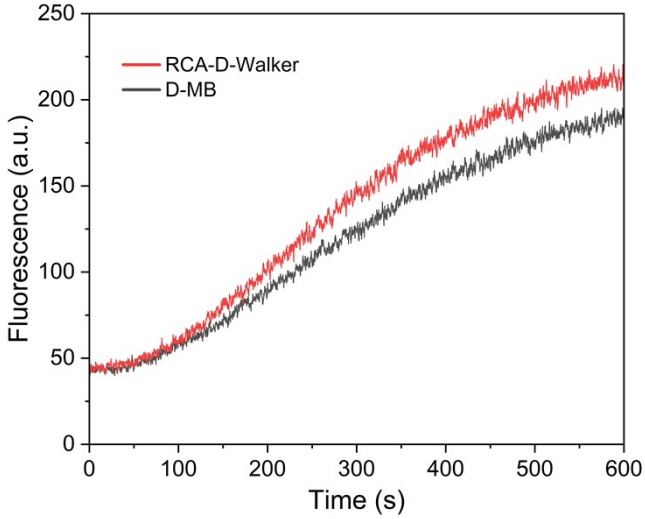


Figure S4. The dynamic evaluation of RCA-D-Walker and D-MB in response to D-miR-21.

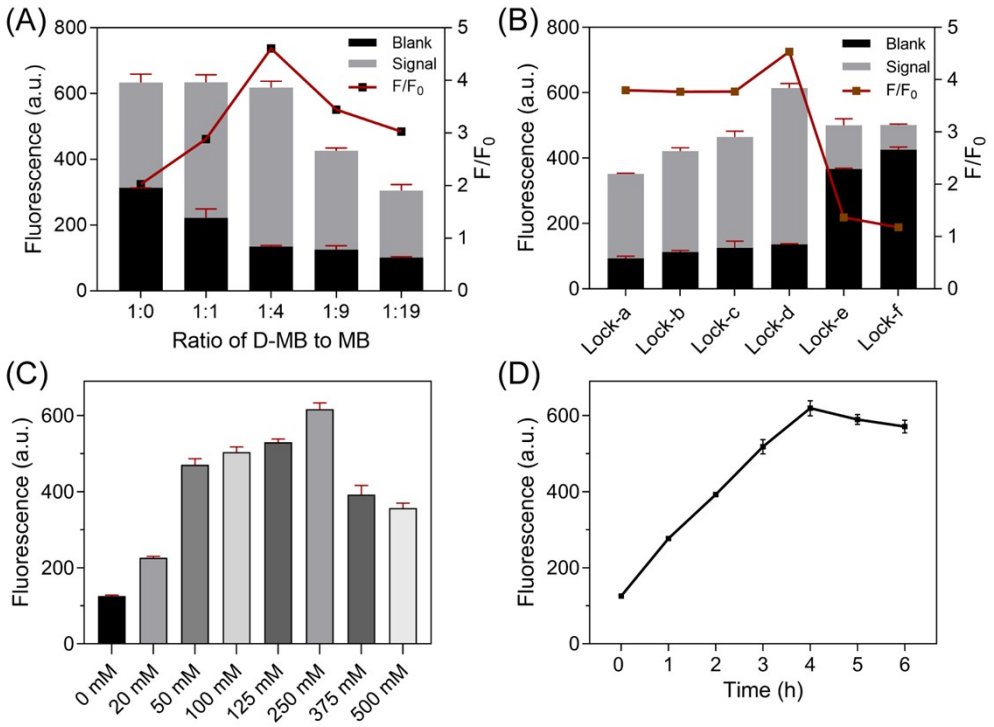


Figure S5. Optimization of the design and detection conditions of RCA-D-Walker. (A) Fluorescence intensity of RCA-D-Walkers assembled with a series of ratios of D-MB and MB in response to D-miR-21. (B) Fluorescence intensity of RCA-D-Walkers assembled with lock strands of varying lengths in response to D-miR-21. For (A) and (B), relative fluorescence intensity was calculated by F/F_0, where F and F_0 represent the fluorescence intensity induced by target D-miR-21 and blank, respectively. (C) Fluorescence intensity of RCA-D-Walker in response to D-miR-21 at different concentrations of Na^+. (D) Fluorescence changes when RCA-D-Walker is incubated with D-miR-21 for various amounts of time. Error bars represent the results from three parallel experiments.

Experimental procedure:

D-MB and MB were mixed at different molar concentration ratios (1:0, 1:1, 1:4, 1:9, 1:19), then mixed with 1 μL of RCA-p, 2 μL of Linker and adjusted to a total volume of 40 μL with TAMg. After thorough mixing, incubate for 1 hour at room temperature to obtain RCA-D-Walker solution (40 μL, 0.5 μM). Each proportion of RCA-D-Walker was mixed sequentially with 1 μL of D-miR-21, followed by the addition of 10 μL of NaCl, and diluted to 200 μL with TAMg. The mixture was then incubated at 37°C for 4 h, which fluorescence signals were detected according to the experimental conditions described in "**SA.6 Fluorescence detection**". Blank control group was used with 1 μL ddH_2O instead of D-miR-21, and other steps were the same. Following the experiments, the lengths of the locked strand, the concentrations of the cofactor Na^+, and the incubation times were systematically varied to optimize the experimental conditions.

Discussion:

Since our detector is designed based on a DNAzyme, it's important to consider that insufficient DNAzyme may lead to competition between probes, thereby reducing RCA-D-Walker reaction efficiency. Conversely, an excess of DNAzyme could interfere with signal transmission, resulting in decreased detection performance. Therefore, optimizing the ratio of D-MB to MB to achieve the best balance is crucial. As shown in Fig S5A, the F/F0 of RCA-D-Walker was maximum and the best performance was achieved only when the ratio of D-MB to MB was 1:4 compared to

other conditions. Therefore, we used this ratio to prepare the RCA- D-Walker.

Based on the design principle we understood that in the absence of D-miR-21, the lock-chain reduces the fluorescence background by binding to the DNAzyme and making it dormant. In order to optimize the length of lock-strand, we evaluated the response signals of the detector for six different lock-strand syntheses. As shown in Fig S5B, clearly, the RCA-D-Walker assembled with lock-d exhibited the strongest signal-to-noise ratio, indicating its effectiveness in silencing the DNAzyme, and thus it was identified as the optimal locking strand.

Given that DNAzyme exhibits higher catalytic activity in the presence of Na^+. Therefore, we investigated the effect of Na^+ concentration on the fluorescence signal as shown in Fig S5C. In the absence of Na^+, little response signal was observed, highlighting the importance of Na^+ as a cofactor for RCA-D-Walker function. Notably, the signal peaked at a Na^+ concentration of 250 mM.

We determined the optimal performance of the detector by exploring the reaction time. As shown in Fig S5D, it is obvious that the fluorescence signal peaks at 4 h of incubation, and therefore, 4 h was chosen as the optimal reaction time.

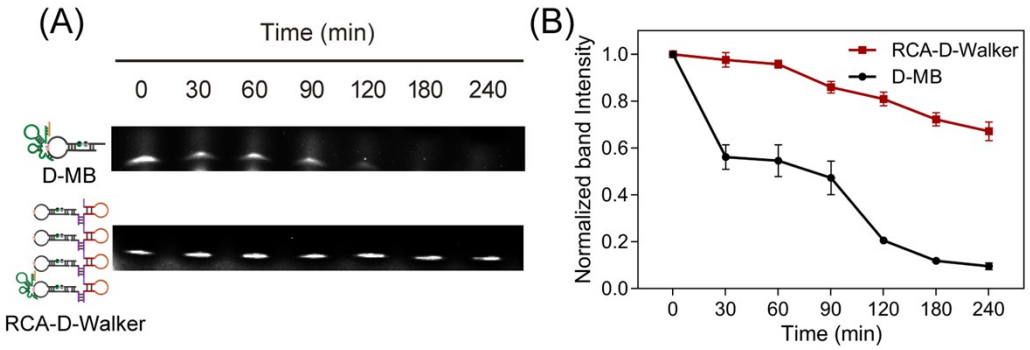


Figure S6. The stability analysis of RCA-D-Walker in cell lysate. (A)The dPAGE analysis of the stability of RCA-D-Walker in HeLa cell lysate. (B) Quantitative evaluation of the fluorescence intensity of the bands shown in (A), error bars represent standard deviations from three independent experiments.

Experimental procedure:

Preparation of Cell Lysate. HeLa cells were grown in a 25 cm^2 culture flask until they reached 80% confluence, then washed with PBS and treated with 500 µL of trypsin. The cells were centrifuged at 900 rpm for 3 min, and the supernatant was discarded.

The cells were then resuspended in PBS, and after removing the PBS, 500 μL of lysis buffer (containing 50 mM Tris-HCl, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% SDS, and protease inhibitor at a 1:100 dilution) was added. The mixture was incubated on ice with shaking for 20 min. Next, the lysate was centrifuged at 12,000 rpm for 30 min at 4°C, and the supernatant was collected and stored at -20°C for future use. D-MB and RCA-D-Walker (0.5 μM) were prepared as described in the section "**SA.2 Construction of the RCA-D-Walker**". 4 μL of each sample were incubated with 4 μL of the cell lysate at 37°C for different time intervals (0, 30, 60, 90, 120, 180, and 240 min).

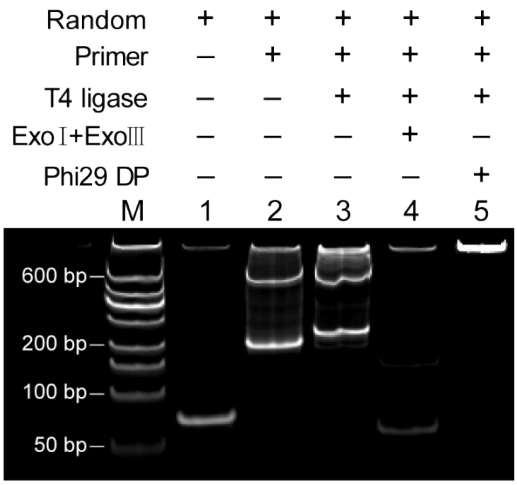


Figure S7. 15% nPAGE analysis of the prepare process of RCA-p-R. T4 DNA ligase, Phi29 DNA polymerase, exonuclease I and Exonuclease III are abbreviated to T4 ligase, Phi29 DP, Exo I and Exo III, respectively.

Experimental procedure:

The experimental operation and process are the same as in Figure 1A, with the only difference being that the Template is replaced with the Random.

Discussion:

In lane 4, the results show that random can also form a loop template with primers with the help of T4 ligase. Lane 5 further confirms the RCA products generated from Random through rolling circle amplification.

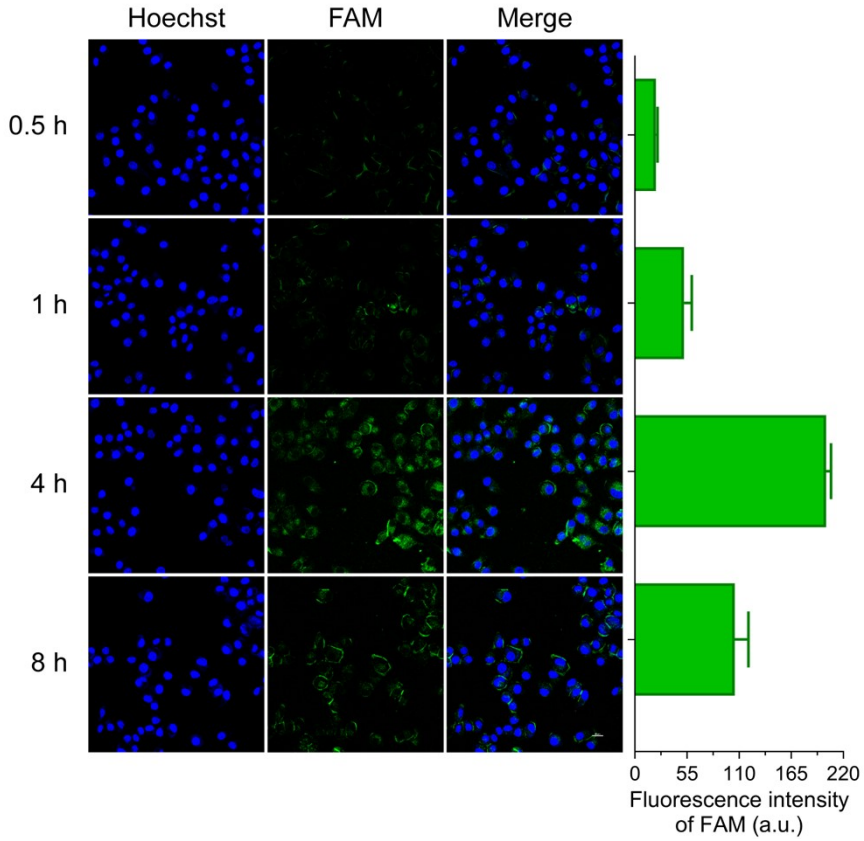


Figure S8. Confocal fluorescence images of HeLa cells incubated with RCA-D-Walker for different times. Scale bar: 20 μm. Right panel: Quantitative analyse of FAM fluorescence by ImageJ in corresponding images. Error bars represent standard deviation from three independent parallel experiments.

Experimental procedure:

HeLa cells was incubated with RCA-D-Walker for 0.5 h, 1 h, 4 h, or 8 h, respectively. After incubation, the cells were washed three times with 500 μL of PBS, followed by fixation with 500 μL of paraformaldehyde for 15 min. Subsequently, the cells were washed with 500 μL of PBS to remove excess paraformaldehyde. Finally, the cells were stained with 500 μL of Hoechst 33342 solution (10 $\mu g \cdot mL^{-1}$) for 15 min, washed again with PBS, and then imaged using confocal microscopy.

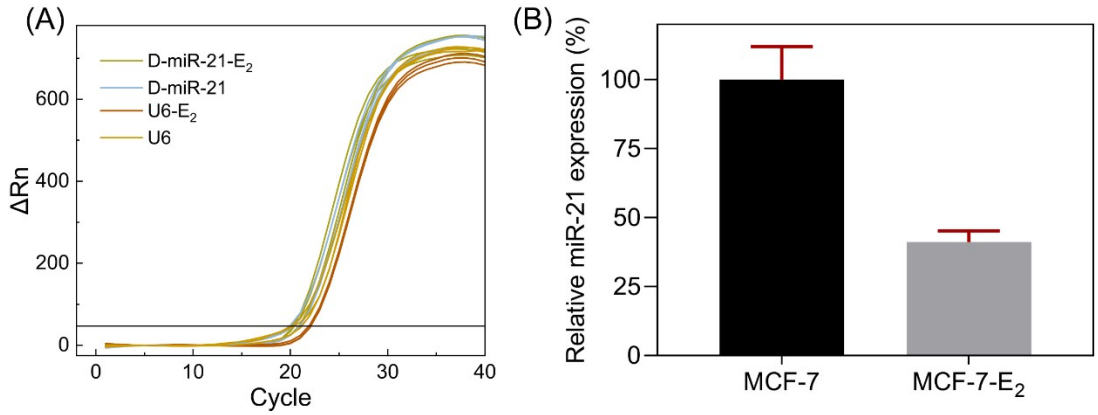


Figure S9. Quantitative RT-PCR analysis of miR-21 from E_2-treated MCF-7 cells. (A) qRT-PCR curves for miR-21 and U6. U6 small RNA as an internal control. The black horizontal line represents threshold line, and the threshold is 50. (B) Quantification of miR-21 expression level. The miR-21 expression in MCF-7 cells defined as 100%. Error bars represent the results from three independent experiments.

4. Section D: References

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