Electronic Supplementary Information (ESI)

Amplification-free miRNA detection with CRISPR/Cas12a System based on Fragment Complementary Activation Strategy

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Materials and Instruments

Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), trisodium citrate, tris-(2-carboxyethyl) phosphine hydrochloride (TCEP), streptavidin (SA), monomethoxy polyethylene glycol thiol (mPEG-SH) and sucrose were purchased from Sigma–Aldrich (St. Louis, MO, USA). EnGen Lba Cas12a and NEBuffer 2.1 were purchased from New England Biolabs (Beijing, China). N-hydroxysuccinimide ester-Cyanine 5 (Cy5-NHS), orthoboric acid and sodium chloride (NaCl) were obtained from Macklin Co., Ltd (Shanghai, China). Phosphate-buffered saline (PBS, 0.01 M, pH 7.4), DNase/RNase-free water, Hoechst 33342 stain solution, bovine serum albumin (BSA) and biological grade dimethyl sulfoxide (DMSO) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Solarbio Science & Technology Co., Ltd. Serum/plasma miRNA extraction kit, HG TaqMan miRNA-21 qPCR Kit and HG TaqMan miRNA qPCR kit were brought from HaiGene Biotech Co., Ltd (Harbin, China). Fetal bovine serum (FBS), Dulbecco'smodified Eagle medium (DMEM), and penicillin-streptomycin solution were purchased from Gibco Co., Ltd (Beijing, China). Cryopreservation medium and trypsin were purchased from New Cell & Molecular Biotech Co., Ltd (Suzhou, China). The sample pads, polyvinyl chloride (PVC) backing cards and absorbent pads were purchased from Shanghai Kinbio Technology Co., Ltd. (Shanghai, China). The nitrocellulose membranes (type: CN140) were purchased from Sartorius Co., Ltd. (Göttingen, Germany). The oligonucleotides were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and their sequences are listed in **Table S1**.

The WM-100 automatic programmable cutter and XYZ-3050 biodot dispensing platform were supplied by Shanghai Kinbio Technology Co., Ltd. (Shanghai, China). The time-based fluorescence data were obtained using a Roche LightCycler96 instrument (USA). Fluorescence images were obtained with a high-resolution laser confocal microscope (CLSM, Leica, Germany). Flow cytometry analysis was performed by a flow cytometer (BD, Fascicular, USA).

Synthesis of the AuNPs

In this assay, the traditional colloidal gold solution with a particle size of 30 nm was prepared using trisodium citrate to reduce $HAuCl_4 \cdot 3H_2O$. Briefly, 49 mg of

trisodium citrate was dissolved in 75 mL of deionized water and heated to boiling temperature. Next, 600 µL of 24 mM $HAuCl_4 \cdot 3H_2O$ was rapidly added to the mixture. The reaction was maintained for about 10 min at boiling temperature, until the color changed to stable and transparent red, then stirred for 15 min. Finally, the obtained solution was allowed to cool at room temperature and stored at 4°C for further use.

Evaluation of AuNPs-PEG cytotoxicity

The cytotoxicity of AuNPs-PEG was evaluated using an MTT cell proliferation and cytotoxicity assay Kit. The U87 or HT22 cells (4×10^3 cells) were first inoculated in seeded into 6-well plastic-bottom plates and incubated at 37°C in 5% CO_2 for 24 h. After the cells were completely adherent, different concentrations of AuNPs-PEG (1, 2, 5, 10, 20, 30, and 40 µM) were added into the 6-well plastic-bottom plates and then incubated with the cells for 24 h. Then, 20 µL MTT solution (5 mg/mL) was added to each well and incubated for 4 h. After discarding the MTT solution, 180 µL of DMSO solution was added to each well and the crystals were fully dissolved by shaking at 37°C for 10 min. Then, the absorbance value at 570 nm was measured via a Microplate Reader. Finally, cell viability was calculated according to the manufacturer's protocol. All the experiments were repeated at least three times.

Analysis of relative expression of miRNA-10b from clinical samples with RT-qPCR

The cDNA was synthesized from the extracted total miRNAs using the HG TaqMan miRNA qPCR kit (HaiGene). In this step, 4 µL of eluted miRNA was first mixed with 1 µL of 5×TaqMan miRNA RT solution A. The RT procedure was 37°C for 30 min and 85°C for 5 min. Then, 2 µL of 10×TaqMan miRNA RT primer, 2 µL of 10×TaqMan miRNA RT solution B and 11 µL of DEPC-treated water were added above solution. The RT procedure was 30°C for 5 min, 55°C for 60 min and 95°C for 5 min. The cDNA solution was diluted with 40 µL DEPC-treated water before being added to the qPCR system. qPCR analysis of miRNA-10b was performed with HG TaqMan miRNA-21 qPCR Kit (HaiGene). The total reaction solution contained 4 µL of 5×Golden HS TaqMan qPCR mix, 1 µL of 20×miRNA TaqMan assay, 2.5 µL of cDNA template and 12.5 µL of RNase-free water. PCR was performed with pre-denaturation at 95°C for 5 min, 40 cycles of 95°C for 10 s and 60°C for 60 s. In brief, the relative expression of miRNA-10b from

serum samples was evaluated from a standard curve (cycle threshold (Ct) value) for various concentrations of miRNA-10b.

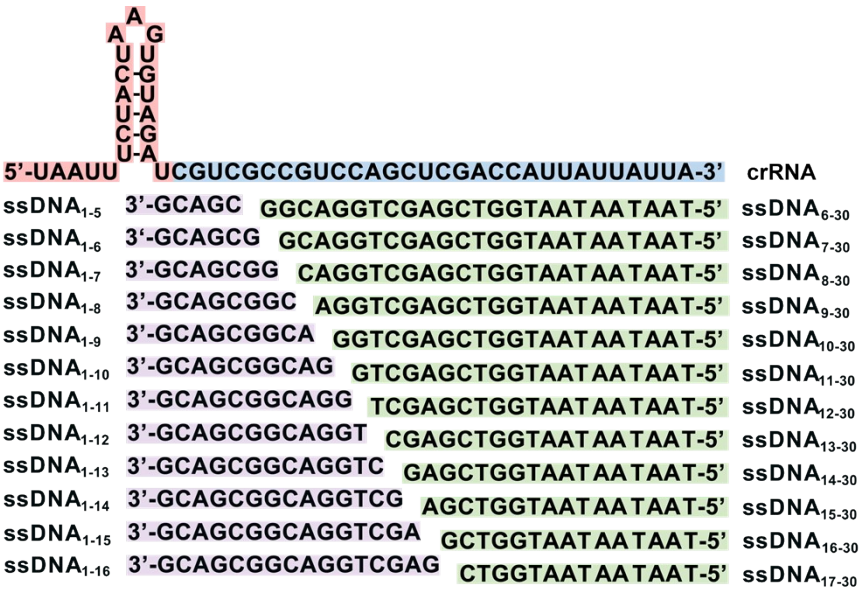

Figure S1. Different nucleotide sequences were used to explore the effect of fragmented ssDNA activators on CRISPR/Cas12a system trans-cleavage activity.

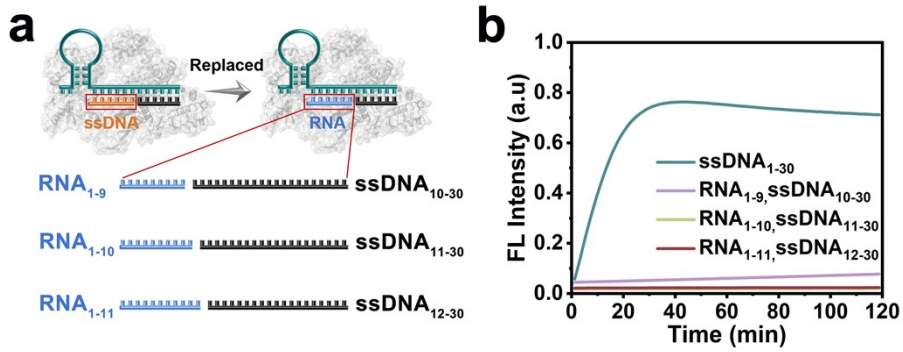

Figure S2. (a) Schematic representation of the CRISPR/Cas12a system activated by inputting pre-ssDNA replaced with pre-RNA. (b) Real-time fluorescence of the Cas12a/crRNA complex binding to fragmented RNA and ssDNA (total 30 nt).

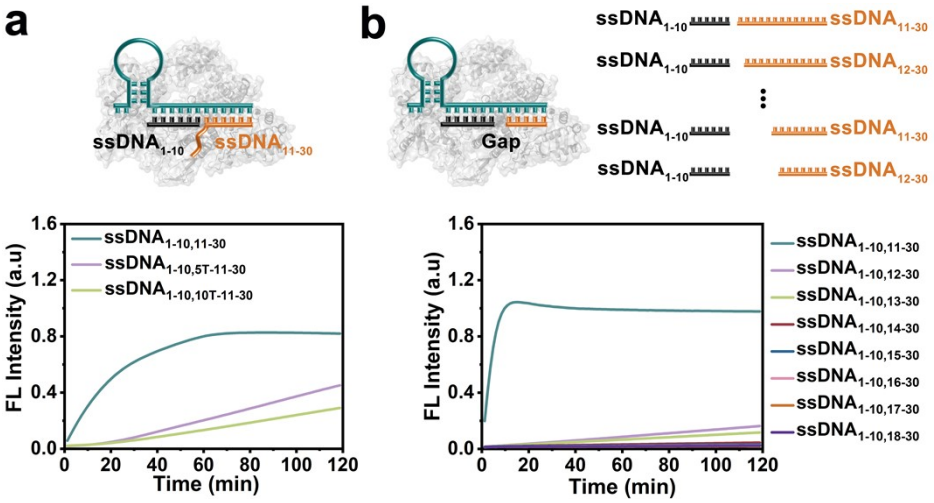

Figure S3. Influence of other structures on the CRISPR/Cas12a system tran-cleavage activity. Schematic and real-time fluorescence curves showing the effects of (a) lengths of 3' protruding termini and (b) deletions on different bases. The experiments were independently repeated three times.

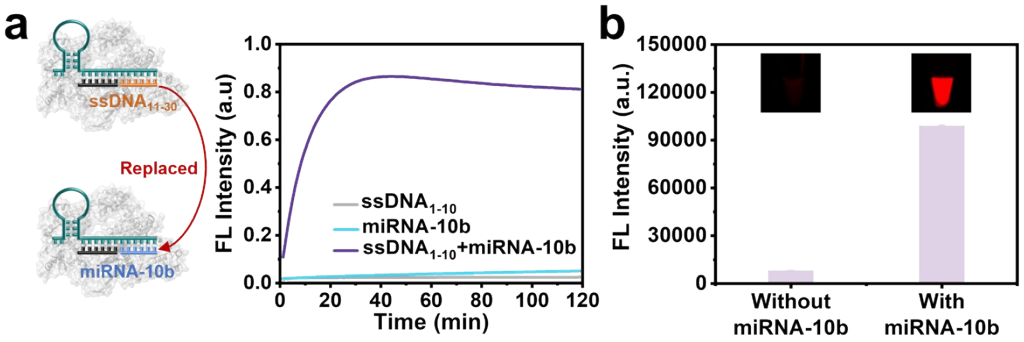

Figure S4. (a) Schematic and real-time fluorescence curves of the FCAS-based CRISPR/Cas12a system trans-cleavage activity reaction, replacing input $ssDNA_{11\text{-}30}$ with miRNA-10b. (b) Corresponding liquid phase analysis (inset: photographs of feasibility results for detecting miRNA-10b).

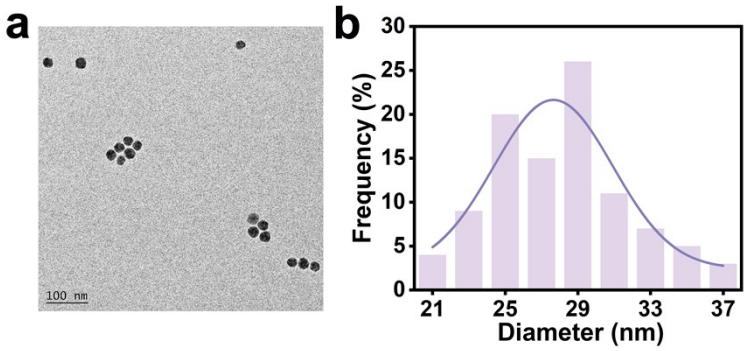

Figure S5. TEM images and size distributions of the prepared AuNPs, respectively.

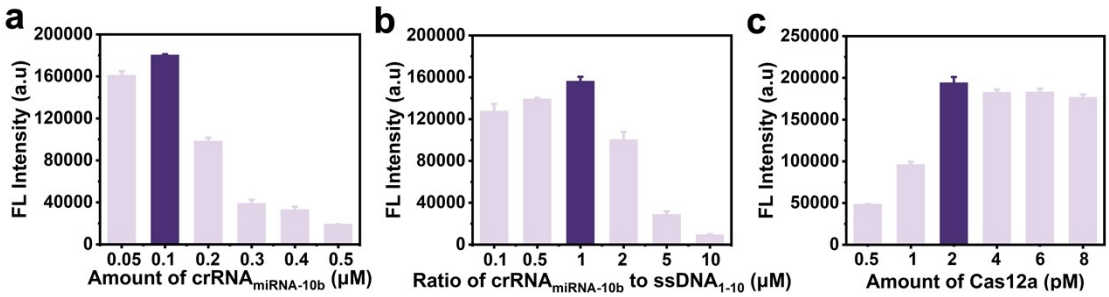

Figure S6. Optimization of experiment parameters for detecting miRNA-10 using the FCAS-based CRISPR/Cas12a system. Effects of (a) the amount of $crRNA_{miRNA-10b}$, (b) the ratio of $crRNA_{miRNA-10b}$ to $ssDNA_{1-10,}$ and (c) the amount of Cas12a proteins on the FCAS-based CRISPR/Cas12a system. Error bars represent the standard deviation of triplicates.

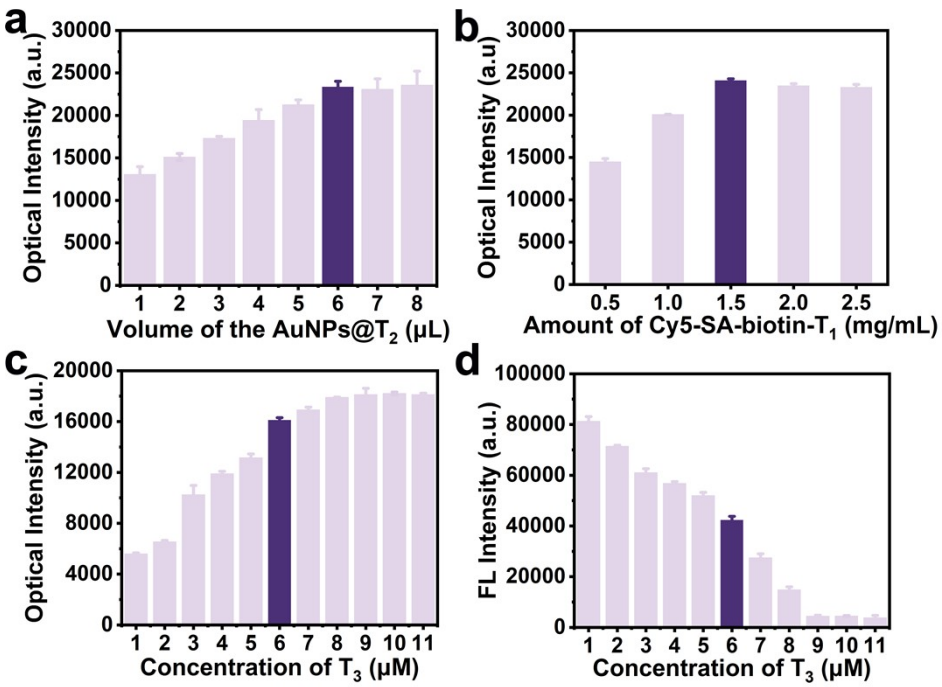

Figure S7. Optimization of experimental parameters for rFLTS. Effects of (a) the volume of the AuNPs@T_2 probes and (b) the amount of Cy5-SA-biotin-T_1 on the test strips. Effects of T_3 reporters concentration on the test strips as observed by (c) the naked eye and (d) the fluorescence. Numbers 1-11 represent T_3 reporters concentrations of 0.001, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2, and 5 μM, respectively. Error bars represent the standard deviation of triplicates.

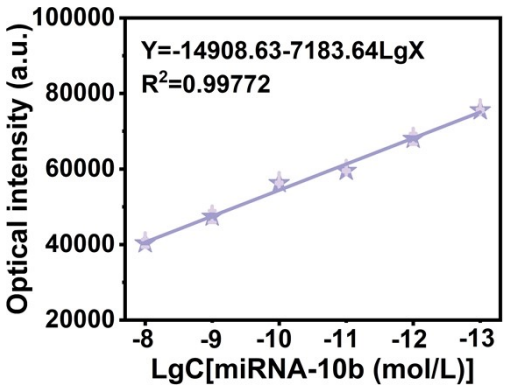

Figure S8. Calibration curve showing the relationship between T-line optical intensity and miRNA-10b concentrations in the range of 10^{-13} mol/L to 10^{-8} mol/L.

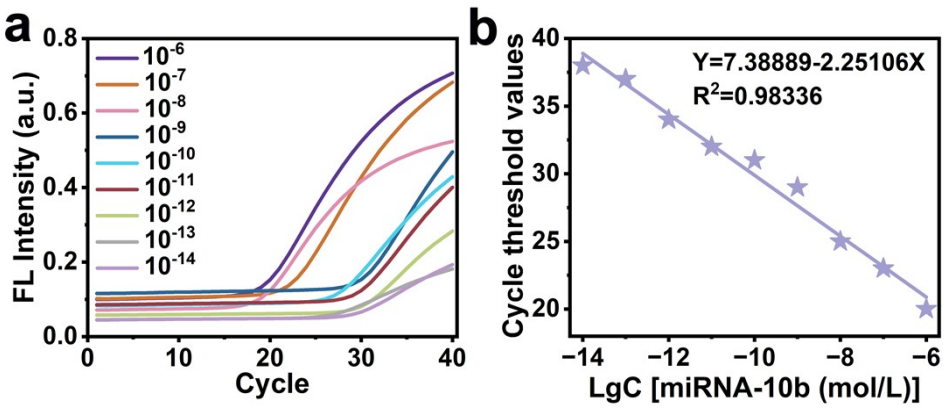

Figure S9. Calibration of the RT-qPCR assay for miRNA-10b detection. (a) Real-time fluorescence RT-qPCR curves for samples converted to cDNA before amplification. (b) Standard curve of cycle threshold versus the logarithmic concentrations of miRNA-10b.

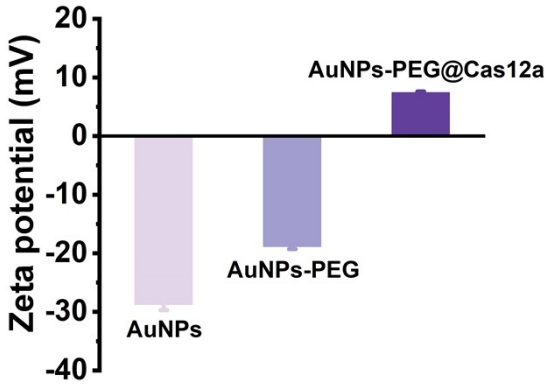

Figure S10. ζ-potentials of AuNPs, AuNPs-PEG, and AuNPs-PEG@Cas12a, respectively.

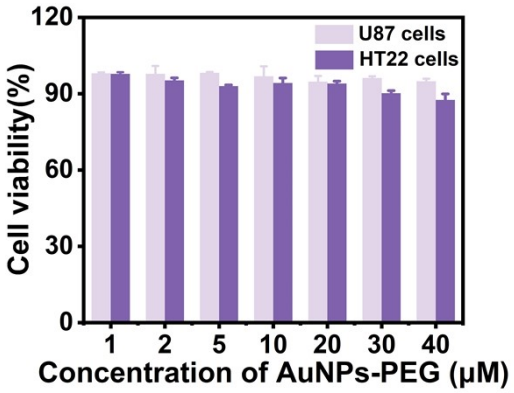

Figure S11. Cytotoxicity of AuNPs-PEG with different concentrations (1, 2, 5, 10, 20, 30, and 40 μM) in U87 and HT22 cells, respectively.

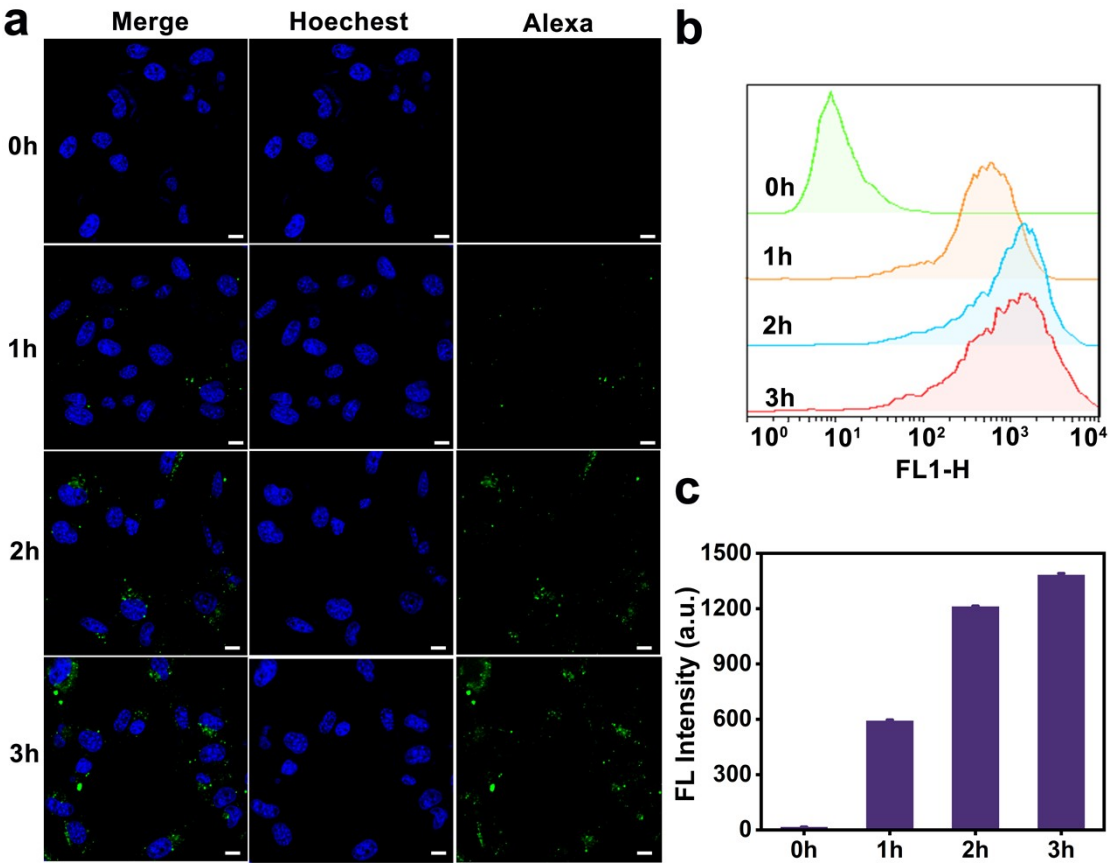

Figure S12. Optimization of cell imaging time. (a) Fluorescence images of cells at different reaction times (scale bar: 10 μm). (b) Flow cytometry results; (c) Statistical histogram of flow cytometry values.

Table S1. The oligonucleotide sequences in this study.

Sequences Name	Sequences（5’-3’）
crRNA	UAAUUUCUACUAAGUGUAGAUCGUCGCCGUCCAGCU CGACCAUUAUUAUUA
$crRNA_{miRNA\text{-}10b}$	UAAUUUCUACUAAGUGUAGAUCGUCGCCGUCCACAAA UUCGGUUCUACAGG
$ssDNA_{1\text{-}30}$	GAGCTGGACGGCGACGTAATAATAATGGTC
$ssDNA_{1\text{-}5}$	CGACG
$ssDNA_{1\text{-}6}$	GCGACG
$ssDNA_{1\text{-}7}$	GGCGACG
$ssDNA_{1\text{-}8}$	CGGCGACG
$ssDNA_{1\text{-}9}$	ACGGCGACG
$ssDNA_{1\text{-}10}$	GACGGCGACG
$ssDNA_{1\text{-}11}$	GGACGGCGACG
$ssDNA_{1\text{-}12}$	TGGACGGCGACG
$ssDNA_{1\text{-}13}$	CTGGACGGCGACG
$ssDNA_{1\text{-}14}$	GCTGGACGGCGACG
$ssDNA_{1\text{-}15}$	AGCTGGACGGCGACG
$ssDNA_{1\text{-}16}$	GAGCTGGACGGCGACG
$ssDNA_{6\text{-}30}$	TAATAATAATGGTCGAGCTGGACGG
$ssDNA_{7\text{-}30}$	TAATAATAATGGTCGAGCTGGACG
$ssDNA_{8\text{-}30}$	TAATAATAATGGTCGAGCTGGAC
$ssDNA_{9\text{-}30}$	TAATAATAATGGTCGAGCTGGA
$ssDNA_{10\text{-}30}$	TAATAATAATGGTCGAGCTGG
$ssDNA_{11\text{-}30}$	TAATAATAATGGTCGAGCTG
$ssDNA_{12\text{-}30}$	TAATAATAATGGTCGAGCT
$ssDNA_{13\text{-}30}$	TAATAATAATGGTCGAGC
$ssDNA_{14\text{-}30}$	TAATAATAATGGTCGAG
$ssDNA_{15\text{-}30}$	TAATAATAATGGTCGA
$ssDNA_{16\text{-}30}$	TAATAATAATGGTCG
$ssDNA_{17\text{-}30}$	TAATAATAATGGTC
$ssDNA_{18\text{-}30}$	TAATAATAATGGT
$RNA_{1\text{-}9}$	ACGGCGACG
$RNA_{1\text{-}10}$	GACGGCGACG
$RNA_{1\text{-}11}$	GGACGGCGACG
$RNA_{10\text{-}30}$	UAAUAAUAAUGGUCGAGCUGG
$RNA_{11\text{-}30}$	UAAUAAUAAUGGUCGAGCUG
$RNA_{12\text{-}30}$	UAAUAAUAAUGGUCGAGCU
$5T\text{-}DNA_{11\text{-}30}$	TAATAATAATGGTCGAGCTGTTTTT
$10T\text{-}DNA_{11\text{-}30}$	TAATAATAATGGTCGAGCTGTTTTTTTTT

Cy5-ssDNA-BHQ2	Cy5-TTTTTTTTTT-BHQ2
Alexa Fluor 488-ssDNA-BHQ1	Alexa Fluor 488-TTTTTTTTTT-BHQ1
miRNA-10b	UACCCUGUAGAACCGAAUUUGUG
miRNA-5010	AGGGGGAUGGCAGAGCAAAAUU
miRNA-21	UAGCUUAUCAGACUGAUGUUGA
miRNA-31	AGGCAAGAUGCUGGCAUAGCU
miRNA-141	CAUCUUCCAGUACAGUGUUGGA
miRNA-122	UGGAGUGUGACAAUGGUGUUUG
T_1	CAACTCACGGTACGA-Biotin
T_2	SH-GGGAACAAACAAGCATTATT
T_3	TCGTACCGTGAGTTGAATAATGCTTGTTTGTTCCC
C_1	Biotin-AATAATGCTTGTTTGTTCCC

Table S2. Comparison of different methods for the detection of miRNA.

CRISPR/Cas12a system combined with other technologies	Methods	miRNA types	LOD	Reference
Endonucleolytically Exponentiated Rolling Circle Amplification (EXTRA)	Fluorescence	miRNA-21	1.64 fM	[1]
Target triggered exponential rolling-circle amplification (T-ERCA)	Fluorescence	miRNA-155	0.31 fM	[2]
Hybridization chain reaction (HCR)	Fluorescence	miRNA-21	75.4 aM	[3]
PolyA-tailing	Fluorescence	miRNA-299	50 fM	[4]
Three-dimensional (3D) DNA nanomachine	Photoelectrochemical	miRNA-141 miRNA-21	5.5 fM 3.4 fM	[5]
Rolling circle amplification and hybridization chain reaction	Fluorescence	miRNA-10b	41 fM	[6]
Dumbbell probe initiated multi-Rolling Circle Amplification (DBmRCA)	Fluorescence	miRNA-200a	5.44 pM	[7]
strand displacement amplification (SDA)	Visual detection on cotton threads	miRNA-let-7a	6.28 pM	[8]
AND logic-gate	Fluorescence	miRNA-205 miRNA-944	36.4 fM	[9]
FCAS-based CRISPR/Cas12a system	**rFLTS**		**5.53 fM**	**This work**

Table S3. Detection recovery rates of miRNA-10b in human serum samples.

Samples	Added (10^{-12} mol/L)	Founded (10^{-12} mol/L) ± SD	Recovery (%)	RSD (%)
1	10	10.88 ± 0.51	108.81	4.69
2	10	11.20 ± 0.55	112.02	4.91
3	10	10.77 ± 0.66	107.77	6.13
4	10	10.84 ± 0.74	108.39	6.83
5	10	11.16 ± 0.32	111.65	2.87

Reference

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