Supporting Information

CRISPR/Cas12a Assay for Amol Level microRNA by Combining Enzyme-free Amplification and Single Particle Analysis

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Reagents.

All oligonucleotides (shown in Table S1) were synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China). The Cas12a was supplied by Tolo Biotech Co., Ltd (Shanghai, China). Sodium chloride solid (NaCl, for acs, ≥99.9%), sodium phosphate dibasic dodecahydrate (Na2HPO4·12H2O, AR, ≥99.9%), sodium phosphate monobasic dihydrate (NaH2PO4·2H2O, ≥ 99.5%), sodium Citrate (98%, Adamas) , and Chloroauric acid hydrate (HAuCl4•xH2O) were obtained from Titan Scientific Co. Ltd (Shanghai, China). Tris-HCl stocking solution (1 M, pH=7.5, sterile), magnesium chloride hexahydrate (MgCl2·6H2O, molecular biology grade), and 10% Tween-20 were all supplied by Sangon Biotechnology Co. Ltd. (Shanghai, China). Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP). The Cas12a proteins and NEBuffer 2.1 buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl2, 100 μg/mL BSA, pH 7.9) were purchased from New England Biolabs (Ipswich, MA, USA). All Native Gel electrophoresis (PAGE) correlated materials which contained ammonium persulfate, acrylamide, N, N,-methylene bis (acrylamide), and N, N, N', N'-tetramethyl ethylenediamine were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). Ultrapure water with 18.24 MΩ cm-1 was utilized from the UPURE Sichuan water purification system. Penicillin-streptomycin solution, culture medium (High glucose), insulin, and Fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc (Fremont, USA). Water used in the RNA-related application was DEPC-treated nuclease-free water purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). Serum samples were provided by Seventh People Hospital Chengdu, Sichuan, China. All cell lines were collected from West China Hospital of Sichuan University, Sichuan, China.

Instruments.

A PerkinElmer NexION 350 quadrupole inductively coupled plasma mass spectrometer was applied throughout the experiment. The detailed working conditions and parameters of the ICPMS apparatus are included in Table S2. The annealing process of preparation of hairpin DNAs was performed with the assistance of K960 Thermal Cycler (Heal

Force Inc., China). Transmission electron microscopy (TEM) characterization was conducted by Tecnai G2 F20 S-TWIN (FEI., USA). The UV-vis spectra for noble metal nanoparticle characterization were carried out by Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Inc). Dynamic light scattering (DLS) and zeta potential for the conformation of noble metal nanoparticle labeling processes were operated by Malvern Zetasizer Nano ZS90 (Malvern PANalytical Ltd., Shanghai, China). The Fluochem M (Cell Biosciences, Santa Clara, CA) was introduced for the electrophoresis imaging experiment.

Synthesis of 25nm Gold nanoparticles.

Based on the previously remarkedly reported synthesizing approaches of the citrated-protected AuNPs with slight modification, we successfully AuNPs with diameters of ~25nm. [1,2] All glass apparatus was thoroughly washed with ultrapure water, then sank into aqua regia for 30 min. Later, 60 ml 0.01% $HAuCl_4$ (w/v) was heated until boiling in a three-necked flask. After boiling it for 20 min, 1200 μL freshly prepared 1% trisodium citrate solution was rapidly pipetted into the above boiling $HAuCl_4$ solution. After boiling and reflexing for another 20 min, the pale-yellow solution was changed to a deep magenta color in about 15 s. Succeedingly, the solution was allowed to slowly cool down to room temperature and stored under 4 °C for later use.

Gold nanoparticle concentrations determination.

An external standard method was introduced for measuring the metal-ion concentrations of the above synthesized and AuNPs-DNAs after digesting by aqua regia (v_{HNO3}: v_{HCl} = 3:1). According to our previous work and reported literature, the molar concentrations of AuNPs and AuNPs-DNAs were calculated by using equation listed below[3]:

$$C_{Arm-A/B-AuNPs} = \frac{6C_{ion}}{\pi \rho_{Au} N_A D^3}$$

C_{ion} means ion concentration after digestion of nanoparticles after digestion. $C_{Arm-AuNPs}$ represented the concentration of Arm_A/Arm_B-DNA-AuNPs, N_A is the Avogadro constant, ρ_{Au} is the density of gold, and D represents the diameters of AuNPs which characterized by TEM.

Surface Functionalization of AuNPs.

After syn-thesizingAuNPs (the synthetic process is described in the Supporting information), the dual-functionalized (thiol and biotin-modified) ArmA/ArmB-DNAs were utilized to prepare ArmA/ArmB-DNA-AuNPs through a rapid freezing-based labeling approach with mild modification.43 1.5 nmol dual-modified ArmA/ArmB-DNA were treated separately with 10-fold excess molar ratio Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) for 30 min. The TCEP-treated Ar-mA/ArmB-DNA were added to 1 mL pre-centrifuging washed AuNPs (1.60 nM) accordingly. Later, the as-prepared ArmA/ArmB-DNA-AuNPs were set in a refrigerator and allow them to freeze under -20°C overnight. Then, the ArmA/ArmB-DNA-AuNPs were allowed to thaw and be washed by PBS buffer (10 mM PB; 150 mM NaCl; 0.01% Tween-20; pH 7.4) through centrifugation (10 minutes at 4°C, 11000 rpm) to eliminate any excess ArmA/ArmB-DNA. The resulting pellet was resuspended in the same PBS buffer and stored under 4°C for later use.

Hybridization Chain Reaction (HCR).

The HCR reaction is the crucial factor within the experiment. The success of HCR is directly related to downstream sensing ability. Firstly, H1, u-H2, and u-H3 were dissolved in Tris-HCl buffer (20 mM Tris-HCl, 800 mM NaCl, 5 mM KCl). Then, 1 μM H1, 1.5 μM u-H2, and u-H3 were separately set in a polymerase chain reaction (PCR) thermal cycler to initiate the annealing process, starting at 95°C for 5 minutes, followed by gradual cooling at a rate of 1°C/min until reaching 4°C. Subsequently, the resulting hairpin DNAs were mixed at final concentrations of 250 nM H1, 500 nM u-H2, and 500 nM u-H3. Next, different concentrations of miR-21 were introduced to the mixed hairpin DNA solution and incubated at 37°C for 30 minutes for the initiation of the HCR reaction.

Detection of miR-21 based on HCR and Sp-ICP-MS.

Equal concentrations of Cas12a protein and crRNA guide were mixed in commercially available 1× NEBuffer 2.1 buffer; additionally, allowing them to pre-incubate at 37°C for 15 minutes. This facilitates the formation of Cas12a-crRNA ribonucleoprotein complexes (RNPs). After this incubation period, 20 μL of the HCR products and 10 μL of linker DNA

substrates (at a concentration of 2.5 μM) were introduced to the preassembled Cas12a-crRNA RNPs for cleaving activities. After letting the above cleaving reaction process for 30 minutes under 37°C, the reaction temperature was elevated to 65°C for 5 minutes to cease the trans-cleavage. Following this, the same concentrations of ArmA/ArmB-DNA-AuNPs (785 pM) were added and incubated at 50°C for 30 minutes with mild vibration. After thorough dilution, the resulting aggregates of AuNPs in different states are ready for Sp-ICP-MS analysis (detailed detection procedure is listed in Supporting Information).

Native-Polyacrylamide Gel Electrophoresis.

30 mL 12% polyacrylamide gel was prepared and employed in the PAGE experiments. Briefly, the entire 30 mL system is composed of 6 mL 5X TBE buffer (445 mM Tris; 445 mM Boric acid; 10 mM EDTA; pH = 8.0 - 8.6), 15 mL 30% Acr-Bis (30:1) gel, and 12 mL ultrapure water. Next, 150 μL 10% ammonium persulfate (APS) and 15 μL N, N, N′, N′-tetramethylene diamine (TEMED) were added right after being prepared, and the polymerization of the gel started slowly under an ambient environment. Then, every sample was mixed with a loading buffer in the volume ratio of 6:1 before loading into each lane. Electrophoresis was operated at 160-170V voltages for 2h and connected with flowing condensate water. Later, the gel was retrieved and sank into diluted 4S Red Plus, followed by dyeing for 1 h at room temperature with gentle vibration. Finally, Fluochem M was applied to imaging the gel.

Serum Samples Preparation and Spiked Recovery Test.

Based on the obtained serum samples, which include serum A and B, the first step involves subjecting the serum to centrifugation at 14000 rpm under 4°C for 15 minutes. Following centrifugation, the supernatant was carefully collected and subsequently diluted by a factor of 10. Next, samples containing varying concentrations of miR-21 (0 pM, 100 pM, and 500 pM) were spiked to each of the serum samples. Later, the designed method was used to assess miR-21 levels within the serum.

Cell Resuscitation.

Pre-heat a water bath to 37°C and prepare 15 mL centrifuge tubes and culture flasks. Prepare complete culture medium for MCF-7, MCF-10a, HEK293, and HepG2 cells by mixing 45 mL DMEM medium, 5 mL FBS, 50 μL of 10 mg/mL insulin, and 500 μL of penicillin-streptomycin solution (5,000 U/mL). Ever cells proceed in the same way. Firstly, gently agitate the thawed cell cryovial in the water bath until the freezing medium is fully dissolved, then transfer the cells to a 15 mL centrifuge tube containing 3 mL of complete culture medium, mixing thoroughly. Centrifuge at 1000 rpm for 3 minutes, discard the supernatant, add 1 mL of complete culture medium, mix thoroughly, and then transfer to a T25 culture flask containing 5 mL of complete culture medium. Set the above cells in an incubator (incubation conditions are 37 °C, 5% CO_2).

Medium Change.

Change the culture medium every 2-3 days while observing cell density under a microscope. Use a pipette to sidcard the old culture medium, then wash the cells twice by adding 2 mL of calcium- and magnesium-free PBS along the edge of the dish. Add 6 mL of fresh complete culture medium and return to the 37°C, 5% CO_2 incubator for continued culture.

Cell Passaging.

When cell density reaches approximately 80 – 90%, proceed with passaging. Prepare 0.25% trypsin-0.53 mM EDTA, complete culture medium, PBS, T25 culture flasks, and 15 mL centrifuge tubes in advance. First, use pipettes to remove the old culture supernatant. Rinse the cells twice with 2 mL of PBS, then add 1 mL of trypsin and incubate in the incubator for 3-5 minutes. Observe the digestion process under a microscope, then terminate digestion by adding 3 mL of complete culture medium. Gently pipette the cells to achieve a good single-cell suspension. Centrifuge at 1000 rpm for 5 minutes, discard the supernatant, and add 1 mL of complete culture medium, mixing thoroughly. Disperse the cell suspension into three T25 culture flask containing 10 mL of complete culture medium (1:3 passage ratio), mix well, and return to the incubator for continued culture.

Cell Cryo-preservation.

Prepare cryovials in advance and prepare the freezing medium (90% FBS + 10% DMSO). First, digest the adherent cells into a single-cell suspension as described in the passaging procedure. Then, count the cells using an automated cell counter. Finally, centrifuge the cell suspension, add an appropriate volume of freezing medium to the pellet, mix thoroughly, and transfer to cryovials. Place the cryovials in a programmed freezing container and store at -80°C for 6 hours before transferring them to liquid nitrogen for long-term storage.

Table S1. Sequences of all oligonucleotides used in this work

Names	Sequences（5' to 3'）
miR-21	UAGCUUAUCAGACUGAUGUUGA
Hairpin DNA-1	AGTCTAGGAAACTGCGTGGGTTAATCTCGTGCTTGTGTGTTGTGCACTTAACCCA
u-hairpin DNA-2	TTAACCAACGCAGTTTCCTAGACTCAGTGTAGTCTAGGAAACTGCGTG
u-hairpin DNA-3	AGTCTAGGAAACTGCGTTGGTTAATACGCAGTTTCCTAGACTACACTG
Hairpin DNA-1-00	AGTCTAGGAAACTGCGTGGGTTAATCTCGTGCTTGTGTGTTGTGCACTTAACC
Hairpin DNA-1-01	AGTCTAGGAAACTGCGTGGGTTAATCTCGTGCTTGTGTGTTGTGCACTTAACCC
Hairpin DNA-1-02	AGTCTAGGAAACTGCGTGGGTTAATCTCGTGCTTGTGTGTTGTGCACTTAACCCA
Hairpin DNA-1-03	AGTCTAGGAAACTGCGTGGGTTAATCTCGTGCTTGTGTGTTGTGCACTTAACCCAC
Hairpin DNA-1-04	AGTCTAGGAAACTGCGTGGGTTAATCTCGTGCTTGTGTGTTGTGCACTTAACCCACG
crRNA	UAAUACGACUCACUAUAGGGUAAUUUCUACUAAGUGUAGAUCUAGACUACACUGAGUCUAG
Arm-A	HS-AAA AAA AAA ACC TCA CCA CCA ACA C
Arm-B	HS-AAA AAA AAA ACA CAC ACA CTC ACA C
Linker	GTG TGA GTG TGT GTG GTG TTG GTG GTG AGG

miR-200C	UAAUACUGCCGGGUAAUGAUG GA
miR-125	UCCCUGAGACCCUAACUUGUGA
miR-342	UCUCACACAGAAAUCGCACCCGU
miR-34	UGGCAGUGUCUUAGCUGGUUGU
miR-9	UCUUUGGUUAUCUAGCUGUAUGA
miR-191	CAACGGAAUCCCAAAAGCAGCUG

Table S2. Working Conditions of sp-ICP-MS

Instrument Parameters.

Parameters	Values
Au (m/z)	197 (100%)
ICP RF Power (W)	1300
Plasma Gas Flow (L/min)	18
Auxiliary Gas Flow (L/min)	1.20
Nebulizer Gas Flow (L/min)	0.96
Deflector Voltage (V)	11.78
Pulse Stage Voltage (V)	1181
Analog Stage Voltage (V)	1917
Sample Uptake Rate (mL/min)	0.25
Dwell Time (μs)	200
Detecting Time (s)	20

Table S3. The ΔG value of the hairpin DNA H1 with different stem lengths

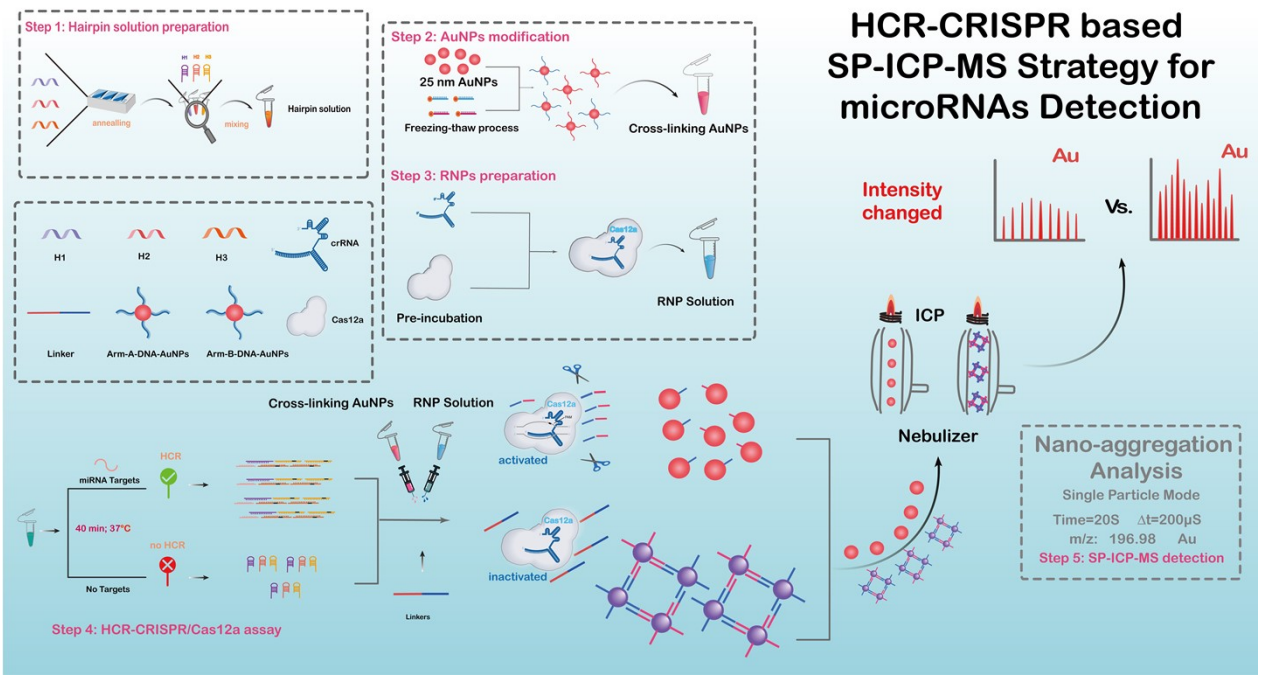
Target	ΔG (kcal/mol)
H1-00	-6.22
H1-01	-8.09
H1-02	-9.06
H1-03	-10.80
H1-04	-12.77

Table S4. Comparison of the analytical performances of different approaches for miRNA targets.

Strategy	Cas effectors	Target	Linear range	LOD	Ref.
Electrochemical Microfluidic	Cas13	miR-20a	10 pM-1 nM	10 pM	[4]
SERS	N/A	miR-21	10 pM-10 nM	2.72 fM	[5]
Electrochemistry	N/A	miR-21	0.5-1000 pM	300 fM	[6]
DSN-Fluorescence	N/A	miR-21	0.5-10 pM	N/A	[7]
MNAzyme-ICPMS	N/A	miR-21	50-1000 pM	10 pM	[8]
Colorimetry	Cas13	miR-17	N/A	500 fM	[9]
Fluorescence	Cas13	miR-17	20-100 fM	1.33 fM	[10]
Sp-ICPMS	Cas12a	miR-21	250 fM-10 nM	220 fM	This work

Table S5. Spiked recoveries in two different Human Serums

Samples	Spiked (pM)	Detected (pM)	Recovery (%)	RSD (%)
	0	Not found	N/A	4.20
Serum A	100	103	103	2.50
	500	501	100.2	12.0
Serum B	0	Not found	N/A	2.70
	100	110	110	1.23
	500	508	102	5.35



Scheme S1. Illustration of Sp-ICP-MS associated HCR-CRISPR/Cas12a nanoparticle analyzing method for miR-21 detection.

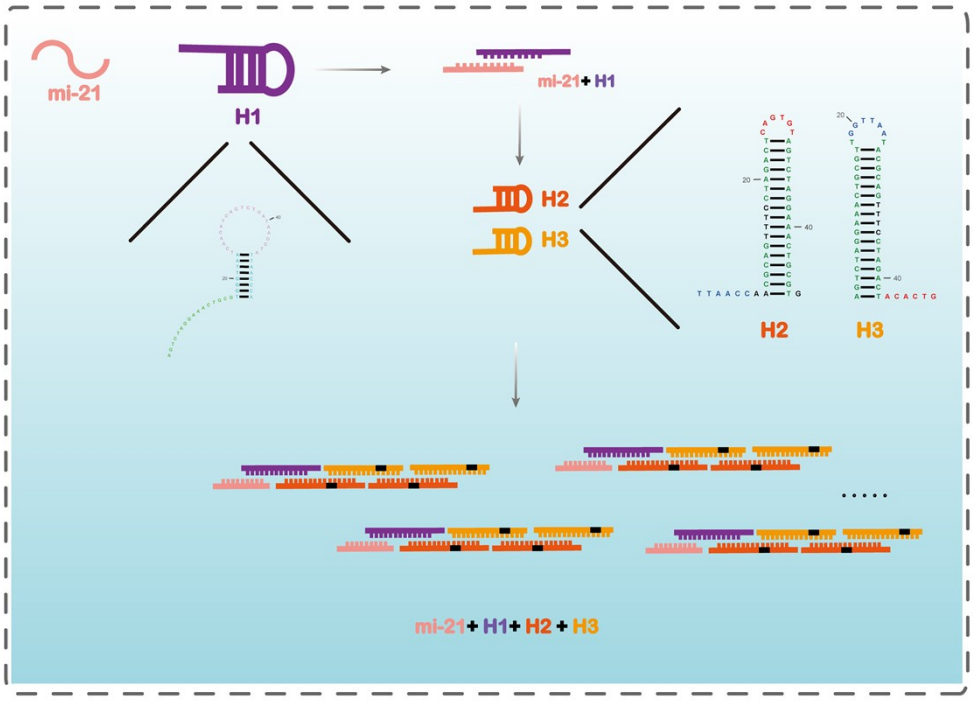


Fig.S1. Illustration of HCR initiating the trans-cleavage of Cas12a detection with the specific structures of hairpin DNA H1, H2, and H3.

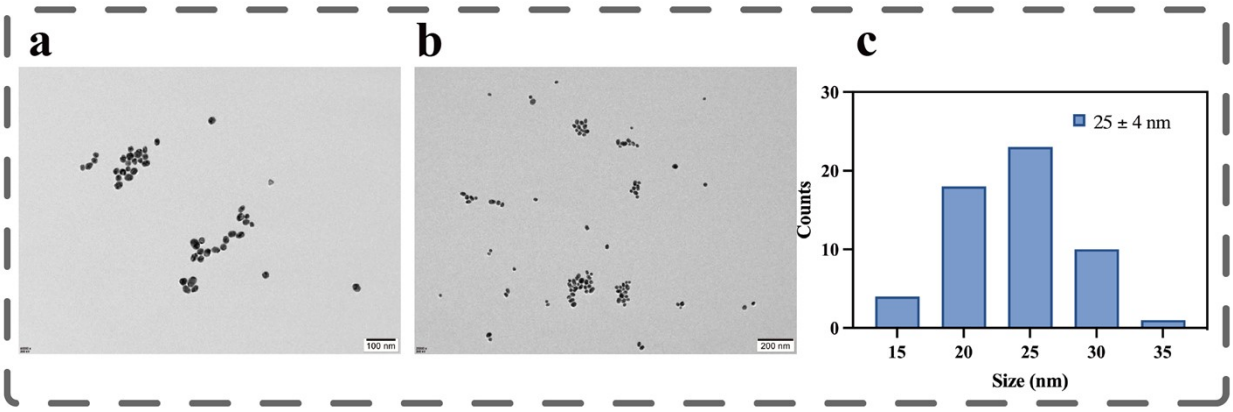


Fig.S2. (a)-(b) are the TEM characterization for AuNPs in the different scale used during this work. (c) demonstrates the size evaluation of AuNPs processed by ImageJ software (1.53a version)

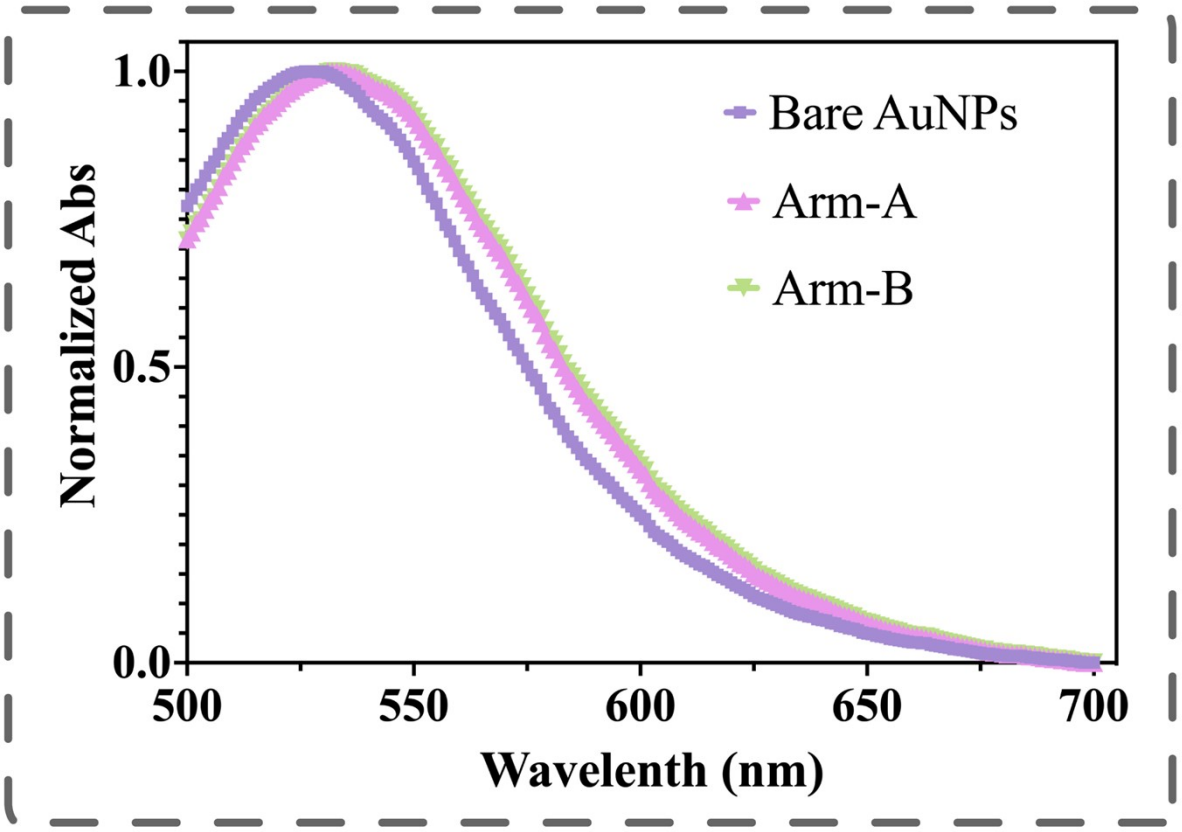


Fig.S3. The Uv-vis spectra characterization of labeling of Arm_A/Arm_B-DNA.

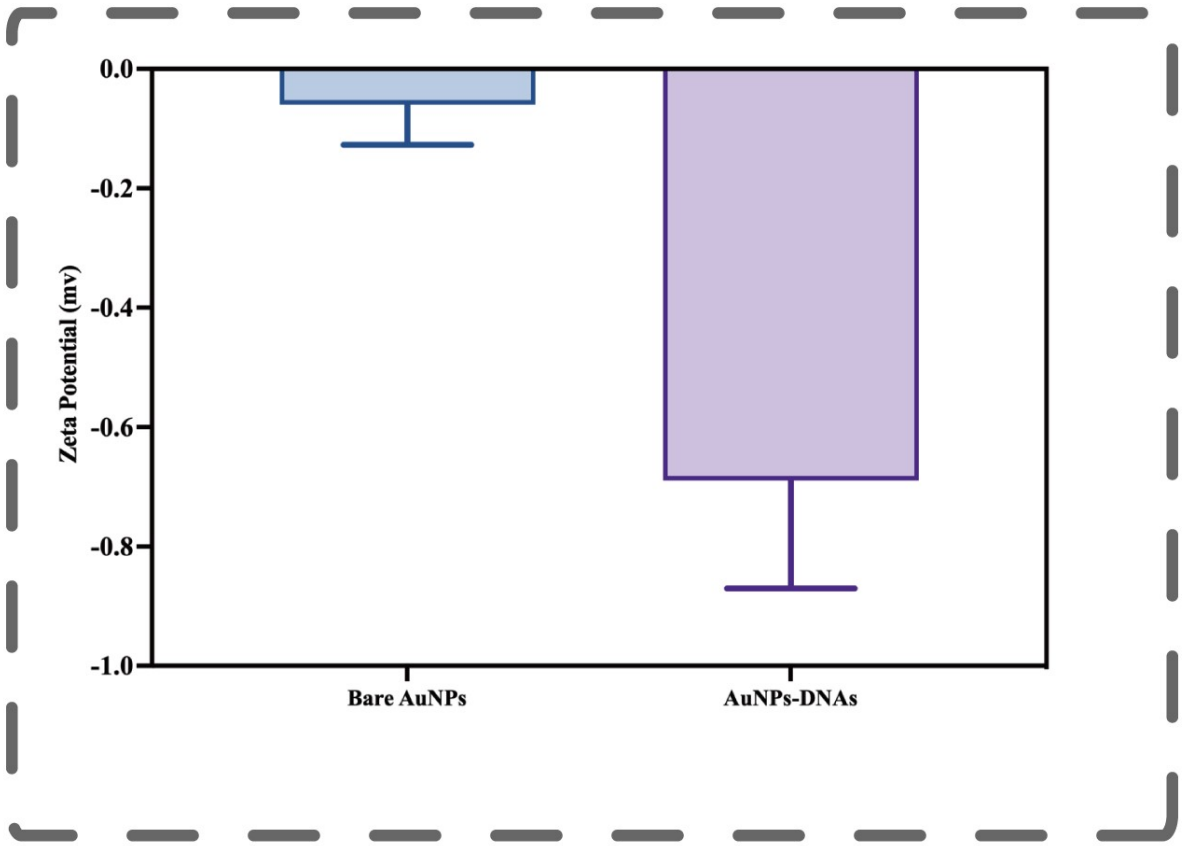


Fig.S4. Zeta potential characterization of labeling of Arm_A/Arm_B-DNA. Mean potential comparison between bare AuNPs and Arm_A/Arm_B-DNA-AuNPs.

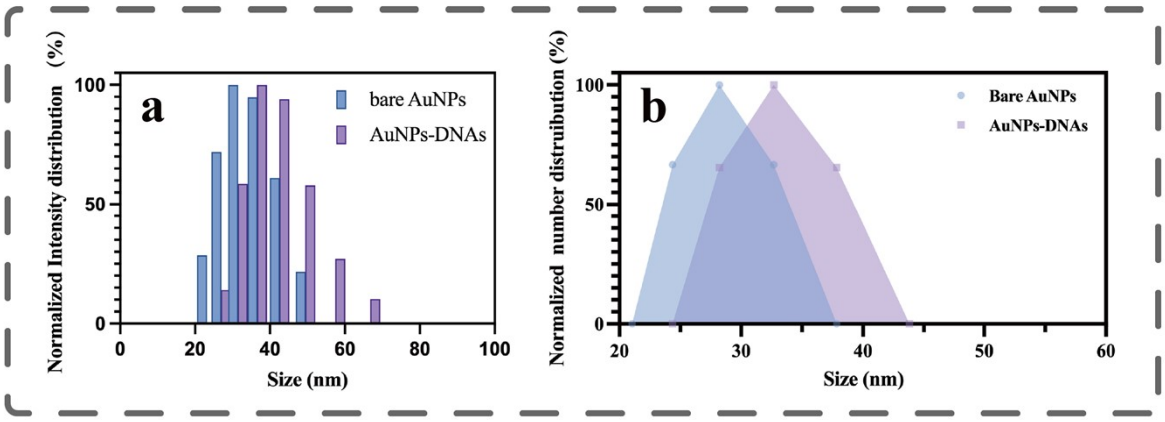


Fig.S5. Characterization of construction of Arm_A/Arm_B-DNA by DLS intensity distribution (left) and number distribution (right)

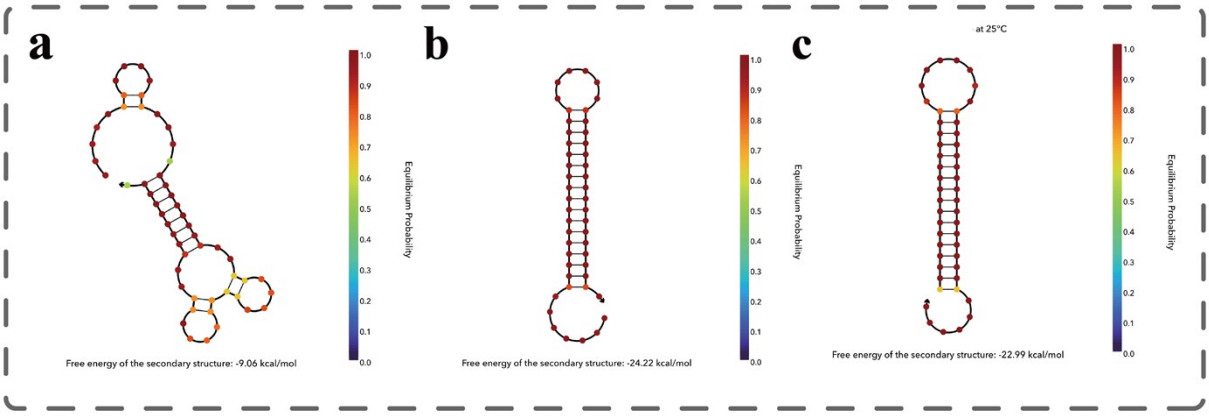


Fig.S6. NUPACK stimulation analysis of the hairpin DNA H1, H2, and H3. (a) Theoretical simulation of H1 (1 μM), (b) theoretical stimulation of H2 (1 μM); (c) theoretical stimulation of H3 (1 μM)

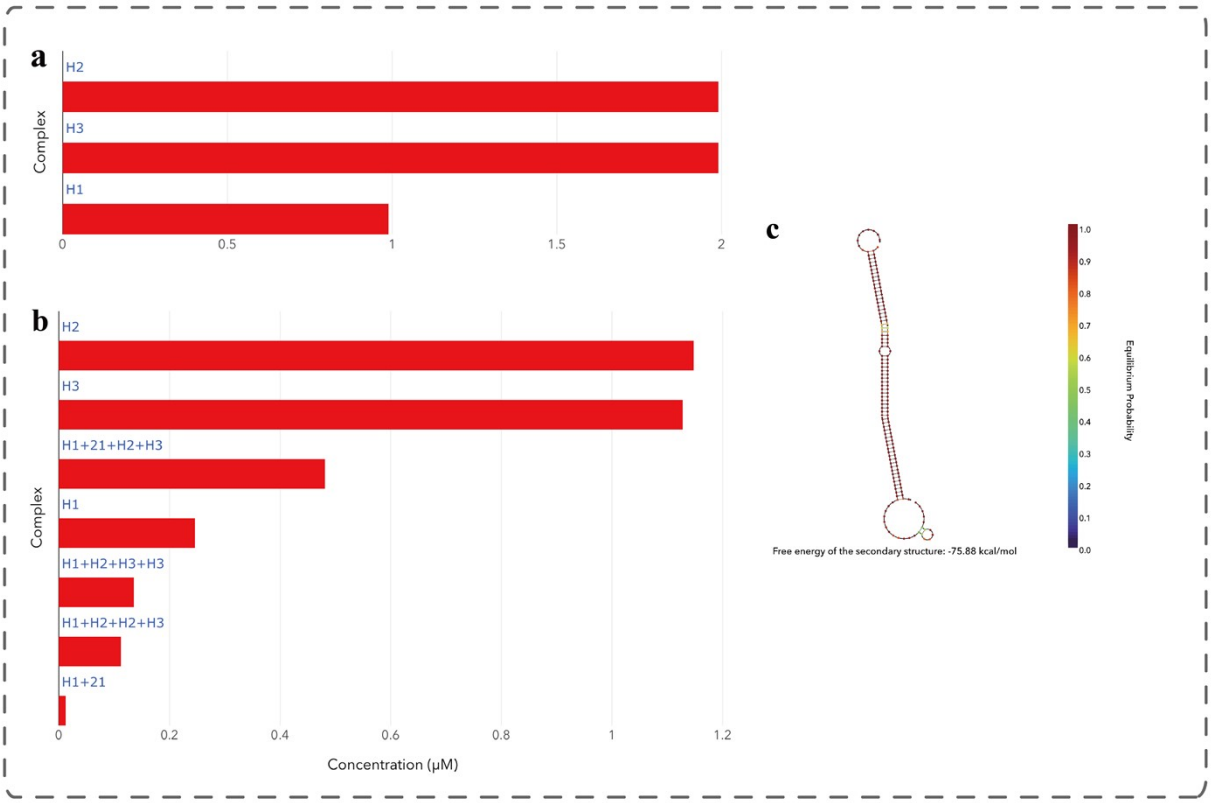


Fig.S7. The theoretical simulations of solution with 1μM H1, 2 μM H2, 2 μM H3 (a); the theoretical simulations of solution with 1μM H1, 2 μM H2, 2 μM, and 500 nM miR-21 (b); the theoretical structure stimulations of HCR reaction (c).

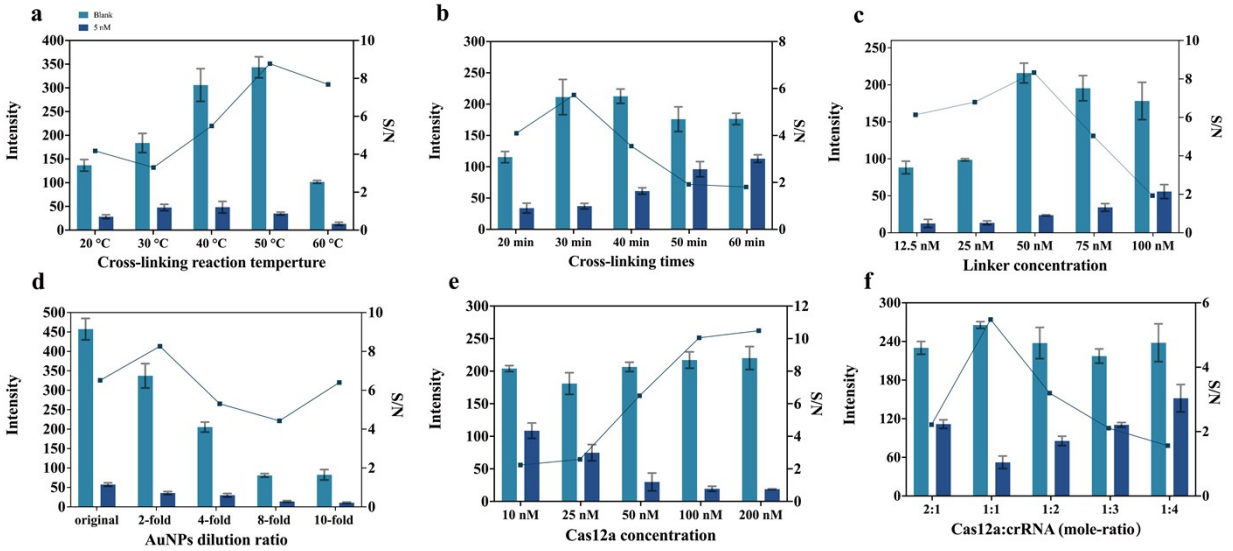


Fig.S8. Optimization of Cross-linking reaction temperature (a); the time of the Cross-linking reaction (b); the concentration of the linker DNAs (c); the dilution ratio of ArmA/ArmB-DNA-AuNPs (d); the concentration of the Cas12a protein (e); the molar ratio of Cas12a:CrRNA (f).

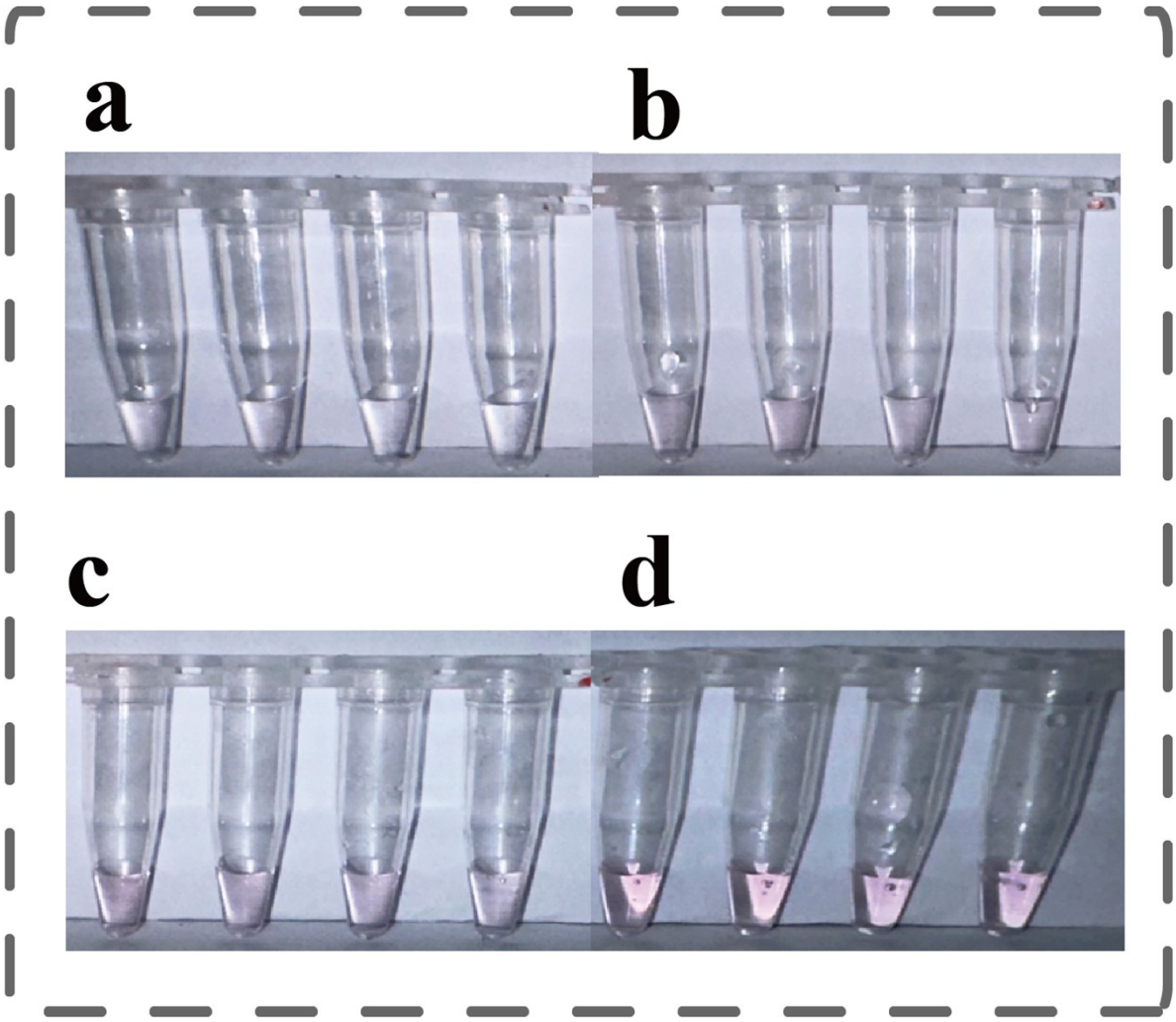


Fig.S9. The Colormetri effect of HCR-CRISPR/Cas12a nucleic acid detection method with the different concentrations of miR-21 0 pM (a); 100 pM (b); 1000 pM (c); 10 nM (d).

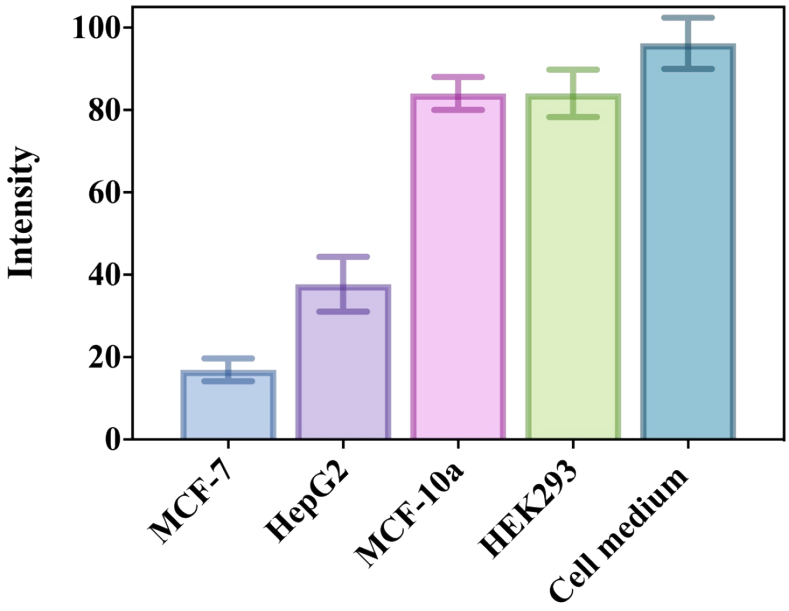


Fig.S10. Analysis of the miR-21 expression levels by using total RNA extracted from different cells.

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