Supplementary Information

Regulating CRISPR/Cas12a trans-cleavage on the hairpin DNA-MBs nanointerface for enhanced multiplexed sensing application

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Table of Contents

Experimental Procedures

Reagents and Materials

Cas12a (Cpf1) was purchased from New England Biolabs Inc. Streptavidin-coated magnetic beads (100 nm/300 nm/1 μm, 10 mg mL^{-1}) were acquired from BioMag Scientific Inc. (Wuxi, China). The CRISPR RNA (crRNA) was synthesized by Takara Biotechnology (Beijing, China). HPLC-purified DNA oligonucleotides were prepared by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China), and all sequences involved were listed in Table S1 (hairpin was abbreviated as HP). Tween-20, Tris, EDTA-2Na, $Ru(bpy)_2Cl_2 \cdot 2H_2O$ and dipyridophenazine (dppz) were purchased from Sigma-Aldrich (Shanghai, China). Nitric acid (HNO_3) was obtained from Jingrui Chemical Co., Ltd. (Suzhou, China). Anhydrous ethanol and methanol, HCl, NaCl, NaOH and $MgCl_2$ were purchased from Chron Chemical Reagent Co., Ltd. (Chengdu, China). SYBR Green I was bought from Yisheng Biotechnology Co., Ltd. (Shanghai, China). 2X high fidelity PCR master mix for PRV DNA amplification was purchased from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China).

All solutions were prepared using ultrapure water (>18.0 MΩ cm^{-1}) produced from a Milli-Q water system. The buffers used were listed as follows:
Reaction buffer: 25 mM Tris-HAc, 10 mM $MgCl_2$, 0.02% Tween-20, and 100 mM NaCl (pH=8.5); binding and washing buffer (B&W buffer): 10 mM Tris-HCl, 1 mM EDTA, 1 M NaCl, and 0.01% Tween-20 (pH=7.4); storage buffer: 10 mM Tris-HCl, 1.25 mM $MgCl_2$, and 0.01% Tween-20 (pH=7.4); 1×CRISPR Buffer: 10 mM Tris-HCl (pH=7.5), 10 mM $MgCl_2$，and 0.02% Tween-20; 10×CRISPR Buffer：100 mM Tris-HCl (pH=7.5), and 100 mM $MgCl_2$.

Apparatus

An iCAP Q inductively coupled plasma mass spectrometer (ICP-MS, Thermo Fisher, United States) was used for the detection of Ru. The operating parameters were summarized in Table S2. Fluorescence measurements were performed using a H1M microplate reader (Biotek, USA). Zeta potential tests were carried out with a Zetasizer

Nano ZS (Malvern, UK). The pH values were measured by a Seven Compact pH meter (Mettler Toledo, Switzerland). Bio-GENER RePure-A gene cycler (Hangzhou, China) was used to carry out the incubation reaction.

Preparation of PRV dsDNA

The conserved sequence of PRV DNA was used as a model target and selected according to the literature, which contained a 5′ TTTG protospacer-adjacent motif (PAM).[1] Two complementary single DNA strands named PRV activator-1 and PRV activator-2 (10 μL, 100 μM) were mixed with 30 μL of reaction buffer and annealed in a PCR thermocycle instrument. For other sequences that require annealing, the buffer and the method used for annealing are the same as above.

Design of crRNA

The crRNA sequence used in this study was designed using the Integrated DNA Technologies website (https://sg.idtdna.com/site/order/oligoentry/index/cpf1). According to the design requirements, the target sequence of crRNA should be 20-24 base. Therefore, the 23-base target DNA sequence containing the Cas12a (Cpf1) PAM site (TTTG) was selected to fully activate Cas12a and achieve maximum *trans*-cleavage activity.

Construction of hairpin DNA-MBs nanointerface

Prior to the conjugation, 100 μL of SA-MBs (10 mg mL^{-1}) was washed three times with and re-suspended in 200 μL of B&W buffer. Then, 5 μL of biotin-terminated hairpin sequence (100 μM) was added to the SA-MBs, which was mixed and shaken at room temperature for 1 h. The resultant hairpin DNA-conjugated MBs were separated from excessive DNA strands by a magnet, rinsed three times with the B&W buffer, and stored at 4 °C in the storage buffer before use.

Activation and verification of the CRISPR-Cas12a system

To activate the CRISPR-Cas12a system, CRISPR RNA (5 μL, 400 nM) was firstly

mixed with Cas12a (5 μL, 400 nM) for 3 min before PRV dsDNA (5 μL, 400 nM), 1× CRISPR Buffer (10 μL) and FQ-Reporter (2 μL, 10 μM) were added. Then, the recovery of fluorescence was monitored using a microplate reader.

Performing HCR in homogeneous solution

To trigger HCR assembly in solution, 2 μL of initiator H0 and two hairpin DNA (H1, H2, 10 μM) was added to sterilized PE tube in sequence, mixed with 92.5 μL of 1×CRISPR Buffer, and incubated at 37 ℃ for 2 h. The HCR assembly among H0, H1 and H2 was also simulated using the NUPACK software (https://www.nupack.org/analysis/input), providing information concerning equilibrium concentration, base-pairing probability, and changes in free energy during HCR.

Performing HCR on the hairpin DNA-MBs nanointerface

HCR assembly at the hairpin DNA-MBs nanointerface resembled that in solution, except that 5 μL of 5 mg/mL MBs-H0 (or MBs-HP) was firstly added to sterilized a PE tube. Afterwards, 2 μL of 10 μM H1 (or H1-FAM) and 2 μL of 10 μM H2 were added. Then, 95.5 μL of 1×CRISPR Buffer was added, vortex mixed, and incubated at 37 ℃ for 2 h.

Polyacrylamide gel electrophoresis (PAGE) analysis

To perform PAGE analysis, 2 μL of 6×loading buffer was mixed with 10 μL of DNA sample and loaded onto the notch of the polyacrylamide gel (12%). Next, the gel was run in 1× TBE buffer under a constant voltage (200 V) for 30 min and stained with the GelRed dye for 10 min. Finally, the ChampGel 7000 automatic digital gel imaging analysis system was used to visualize the electrophoresis result.

Synthesis of $[Ru(bpy)_2dppz]^{2+}$

The ruthenium complex, $[Ru(bpy)_2dppz]^{2+}$, was synthesized according to

literature with slight modifications.[2-4] First, 52 mg of $Ru(bpy)_2Cl_2 \cdot 2H_2O$ and 31.7 mg of dppz were added into the flask with 13 mL of methanol-water mixed solvent inside (volume ratio 1:2). Then, the mixture was heated to and hold at 80 ℃ for 4.5 h under magnetic stirring, yielding a deep red solution. After that, the mixture was concentrated by heating to *ca* one tenth of its original volume. Next, 5 mL of ultrapure water was added to the concentrate. The mixture was boiled for 10 min, cooled to room temperature, frozen in ice bath for 12 h, and filtered to remove the insoluble solids. Subsequently, 1 mL of 10% (m/v) $NaBF_4$ solution was added to the $[Ru(bpy)_2dppz]^{2+}$ solution to form the red brown crude product, $[Ru(bpy)_2dppz](BF_4)_2$. Following suction filtration, the product was collected, dissolved in ethanol, recrystallized, dried in an oven and stored in the dark for use.

Integrating CRISPR-Cas12a *trans*-cleavage and HCR for PRV DNA detection

Firstly, 5 μL of Cas12a (400 nM), 5 μL of crRNA (400 nM), 5 μL PRV DNA (400 nM), 10 μL of 10×CRISPR Buffer and 65 μL of H_2O were mixed. The mixture was incubated at 37℃ for 15 min to fully activate Cas12a. Then, the prepared MBs-HP or MBs-RNA-DNA was added to the mixture, which was further incubated at 37 ℃ for 1 h to perform *trans*-cleavage. After that, the supernatant was removed by magnetic separation and the MBs were washed three times with 1×CRISPR Buffer to isolate from the CRISPR-Cas12a system. Following a heating procedure or otherwise, the MBs were re-suspended in 96 μl of 1×CRISPR Buffer. Finally, 2 μL of H1 or H1-FAM (10 μM) and 2 μL of H2 (10 μM) were added to the above solution, the mixture was vortexed and incubated at 37 ℃ for 1 h to perform HCR at the MBs interface.

After the termination of HCR, the supernatant was discarded, the MBs were gathered, washed three times with 1×CRISPR Buffer, and re-dispersed in 100 or 90 μL of 1× CRISPR buffer depending on the signal output approaches used. Specifically, when FAM-labeled H1 was used, the MBs were suspended in 100 μL of 1× CRISPR buffer and transferred to the microplate for fluorescence recording at one-minute segments (i.e. kinetic curve, Ex=480 nm, and Em=520 nm). The excitation and emission wavelengths were 480 nm and 520 nm, respectively. When unlabeled H1 was

used, the MBs were suspended in 90 μL of 1× CRISPR buffer before 10 μL of 1×SYBR Green I solution or 2 μL of $[Ru(bpy)_2dppz]^{2+}$ (1 mg/mL) were added. In the former case, the obtained mixture was further transferred to the microplate for fluorescence measurements at one-minute segments as well (Ex=480 nm, and Em=520 nm); while in the latter case, the mixture was kept at room temperature for 30 min before transferring to the fluorescence microplate for kinetic curve plotting (Ex=445 nm, and Em=630 nm). After that, the mixture was taken from the microplate, separated by a magnet, washed three times with 1×CRISPR buffer, dissolved with 100 μL of nitric acid (50% v/v) overnight, and diluted to 1 mL for further ICP-MS detection of ^{101}Ru. In this way, the dual-mode FL/ICP-MS detection of PRV DNA was accomplished.

Clinical sample analysis

For sample analysis, the total DNA and PRV-relevant DNA were extracted from the collected blood samples. The target PRV DNA sequence was further amplified using a PCR kit. The specific steps were as follows: 25 μL of high fidelity PCR Master Mix (2×), 2 μL of PCR-PRV-gG-F (10 μM), 2 μL of PCR-PRV-gG-R (10 μM), 2 μL of template DNA and 19 μL of H_2O were mixed at first; the mixture was transferred into the PCR instrument to allow amplification following the program settings described in Table S3; and finally, the PCR amplicons were subjected to FL-ICP-MS dual-mode detection as described above. Animal experiments in this study were approved by the Animal Ethics Committee of Sichuan Agricultural University (20220261). All experimental procedures and animal welfare standards strictly followed the guidelines of Animal Management at Sichuan Agricultural University.

Table S1. Sequences of All oligonucleotides used in this work (HP represents hairpin).

Name	Sequence（5′- 3′）
PRV activator-1	ACGTTTGATCCCGTCCGCCGCCCTTCCGCT
PRV activator-2	AGCGGAAGGGCGGCGGACGGGATCAAACGT
PCR-PRV-gG-F	GCCAGCCGTACACGCAG

PCR-PRV-gG-R	CCGTAGCAGAGCTCCCG
PRV crRNA	UAAUUUCUACUCUUGUAGAUAUCCCGUCCGCCGCCCUUCCGCU
ssDNA-FQ-24C	6-FAM-CCCCCCCCCCCCCCCCCCCCCCCC-BHQ1
HP-FQ-7C	6-FAM-CTAGACTCCCCCCCCCCAGTCTAG-BHQ1
HP-FB-7S	6-FAM-CTAGACTCCCCCCCCCCAGTCTAG-Biotin
H0	AGTCTAGGATTCGGCGTGGGTTAA-Biotin
H0-5C	CCCCCAGTCTAGGATTCGGCGTGGGTTAA-Biotin
H0-10C	CCCCCCCCCCAGTCTAGGATTCGGCGTGGGTTAA-Biotin
H1	TTAACCCACGCCGAATCCTAGACTCAAAGTAGTCTAGGATTCGG CGTG
H1-FAM	TTAACCCACGCCGAATCCTAGACTCAAAGTAGTCTAGGATTCGG CGTG-6-FAM
H1-ROX	TTAACCCACGCCGAATCCTAGACTCAAAGTAGTCTAGGATTCGG CGTG-ROX
H2	AGTCTAGGATTCGGCGTGGGTTAACACGCCGAATCCTAGACTAC TTTG
H2-1	GGATTCGGCGTGGGTTAACACGCCGAATCCTAGACTACT
H2-2	GGATTCGGCGTGGGTTAACACGCCGAATCCTAGACTACTTTG
HP-6	TAGACTCCCCCCCCCCAGTCTAGGATTCGGCGTGGGTTAA-Biotin
HP-8	CCTAGACTCCCCCCCCCCAGTCTAGGATTCGGCGTGGGTTAA- Biotin
HP-10	ATCCTAGACTCCCCCCCCCCAGTCTAGGATTCGGCGTGGGTTAA- Biotin
HP-12	GAATCCTAGACTCCCCCCCCCCAGTCTAGGATTCGGCGTGGGTT AA-Biotin
HP-14	CCGAATCCTAGACTCCCCCCCCCCAGTCTAGGATTCGGCGTGGG TTAA-Biotin
HP-14-FAM	6-FAM- CCGAATCCTAGACTCCCCCCCCCCAGTCTAGGATTCGGCGTGGG

	TTAA-Biotin
HP-18-FAM	6-FAM-CACGCCGAATCCTAGACTCCCCCCCCCCAGTCTAGGATTCGGCGTGGGTTAA-Biotin
HP-21-FAM	6-FAM-ACCCACGCCGAATCCTAGACTCCCCCCCCCCAGTCTAGGATTCGGCGTGGGTTAA-Biotin
HP-24	TTAACCCACGCCGAATCCTAGACTCCCCCCCCCCAGTCTAGGATTCGGCGTGGGTTAA-Biotin
HP-24-FAM	6-FAM-TTAACCCACGCCGAATCCTAGACTCCCCCCCCCCAGTCTAGGATTCGGCGTGGGTTAA-Biotin
HP-24-L0C-FAM	6-FAM-TTAACCCACGCCGAATCCTAGACTAGTCTAGGATTCGGCGTGGGTTAA-Biotin
HP-24-L5C-FAM	6-FAM-TTAACCCACGCCGAATCCTAGACTCCCCCAGTCTAGGATTCGGCGTGGGTTAA-Biotin
HP-24-L15C-FAM	6-FAM- TTAACCCACGCCGAATCCTAGACTCCCCCCCCCCCCCCCCAGTCTAGGATTCGGCGTGGGTTAA-Biotin
HP-24-L20C-FAM	6-FAM- TTAACCCACGCCGAATCCTAGACTCCCCCCCCCCCCCCCCCCCCCAGTCTAGGATTCGGCGTGGGTTAA-Biotin
RNA-DNA	**ttaacccaaattattaa**AGUCUAGGAUUCGGCGUGGGUUAA-Biotin

Table S2. Operation conditions of ICP-MS

Conditions	**Settings**
ICP RF Power	1550 W
Plasma gas flow（Ar）	14 L/min
Auxiliary gas flow（Ar）	0.80 L/min

Nebulizer gas flow（Ar）	1.10 L/min
Isotope monitored	^{101}Ru

Table S3. Program settings for PCR amplification

Step	Temperature	Duration	Cycles
Enzyme activation	95℃	3 min	hold
Denaturation	95℃	15 sec	
Annealing	60℃	15 sec	30
Extension	72℃	30 sec	
Final extension	72℃	5 min	hold

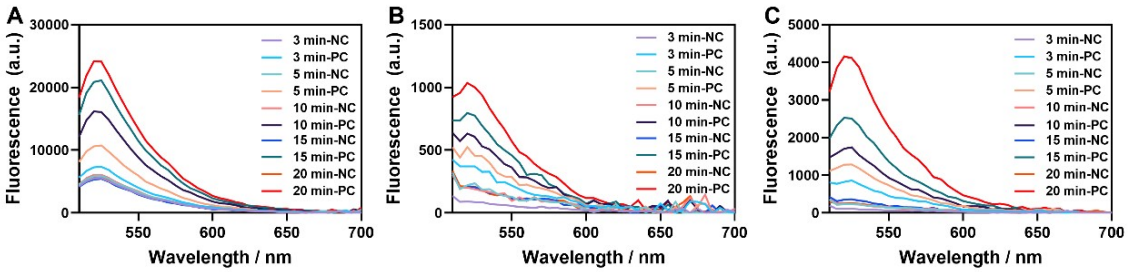

Figure S1. The fluorescence spectra at different cleavage time using (A) ssDNA-FQ, (B) hairpin DNA-FQ and (C) hairpin DNA-FB-MBs as the substrate, respectively.

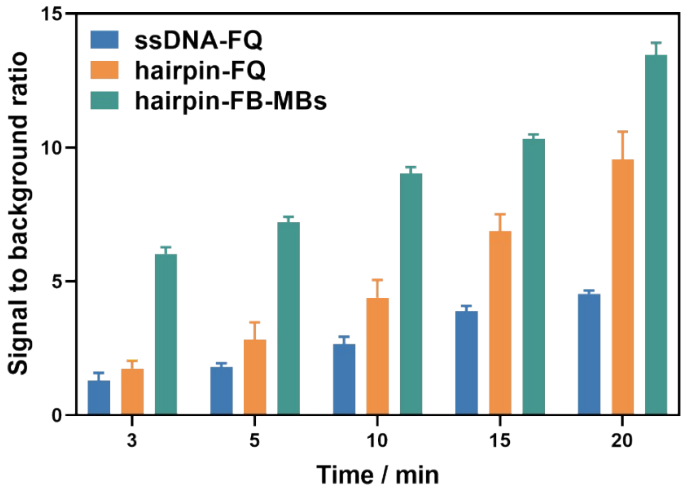

Figure S2. The signal-to-background ratio at different cleavage time using ssDNA-FQ, hairpin DNA-FQ and hairpin DNA-FB-MBs as the substrate, respectively.

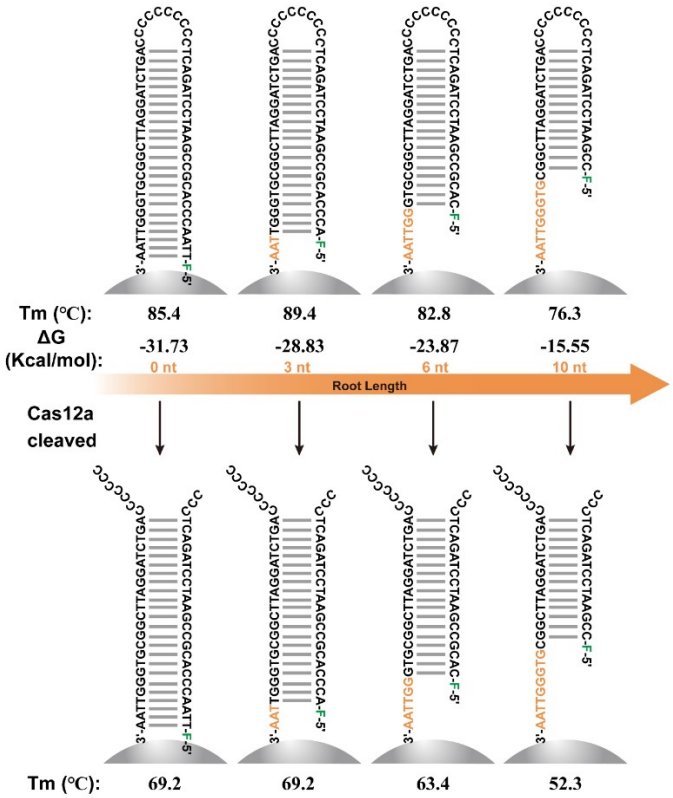

Figure S3. Schematic illustration of hairpin DNA with different root lengths after the loop was cleaved by Cas12a.

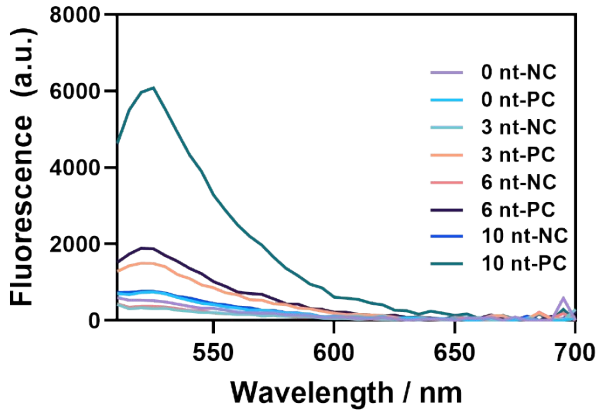

Figure S4. The fluorescence spectra of the supernatant solutions from the cleavage of hairpin DNA-MBs with different root lengths.

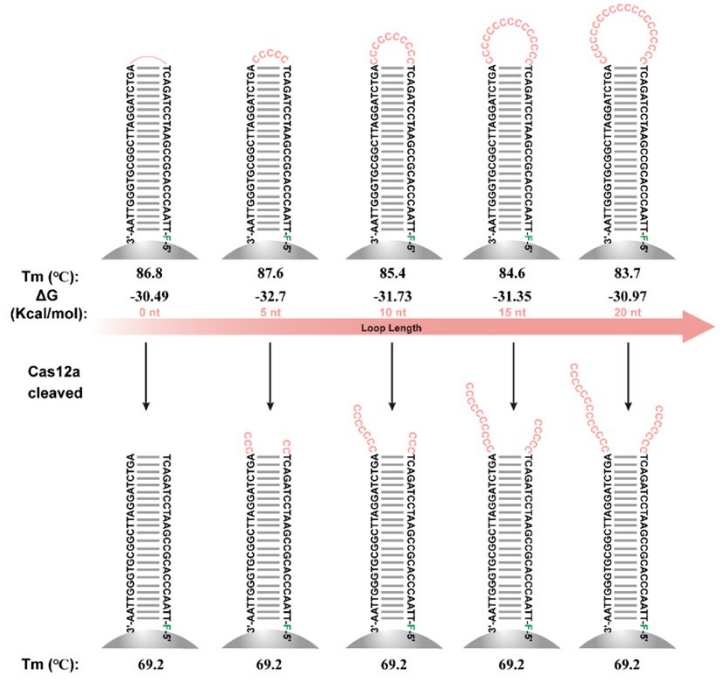

Figure S5. Schematic illustration of hairpin DNA with different loop lengths after the loop was cleaved by Cas12a.

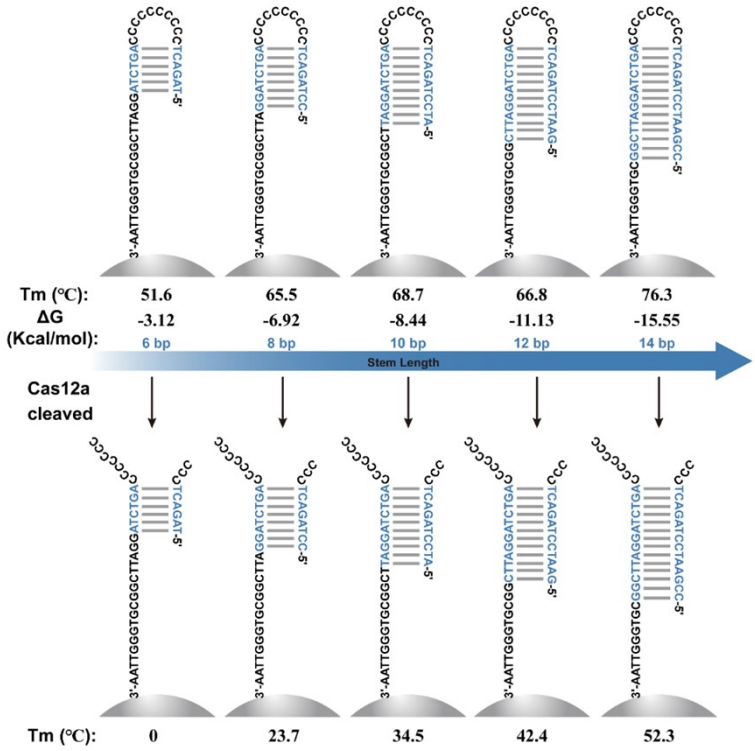

Figure S6. Schematic illustration of hairpin DNA with different stem lengths after the loop was cleaved by Cas12a.

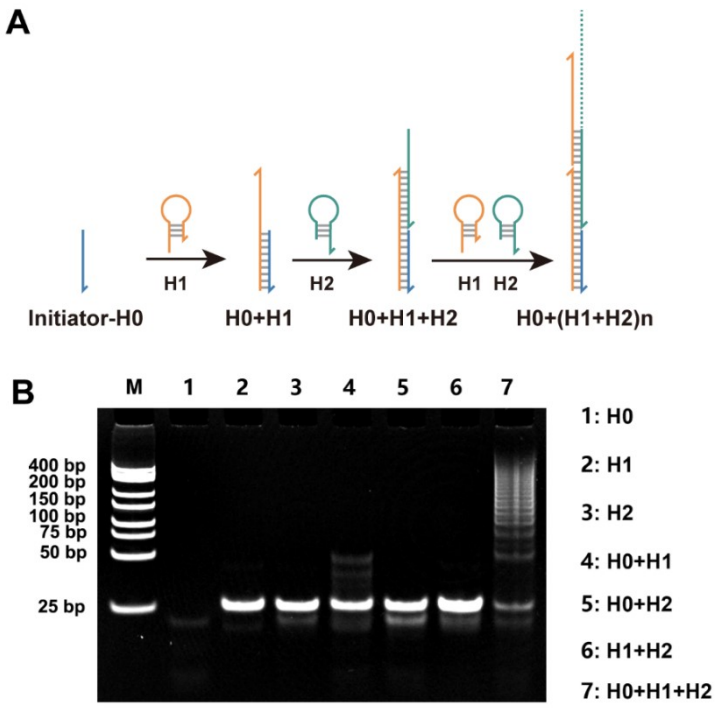

Figure S7. HCR among initiator H0, H1 and H2 in solution: (A) schematic illustration; and (B) polyacrylamide gel electrophoresis analysis of the HCR products.

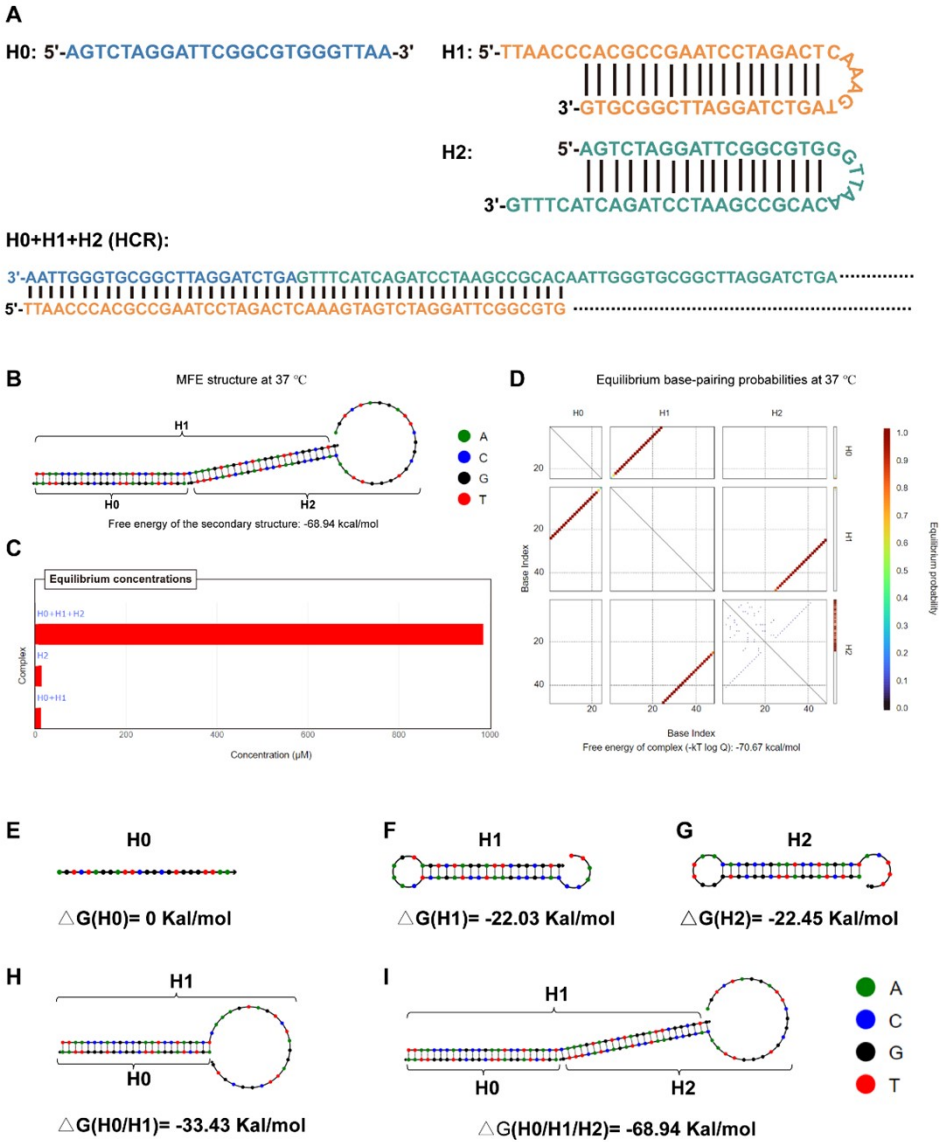

Figure S8. HCR among initiator H0, H1 and H2 in solution simulated by the NUPACK software: (A) sequences of H0, H1 and H2; (B) the sequence of HCR product; (C) equilibrium concentrations analysis; (D) equilibrium base-pairing probabilities at 37 ℃; and the Gibbs free energies of (E) H0; (F) H1; (G) H2; (H) H0+H1; and (I) H0+H1+H2 (HCR assembly).

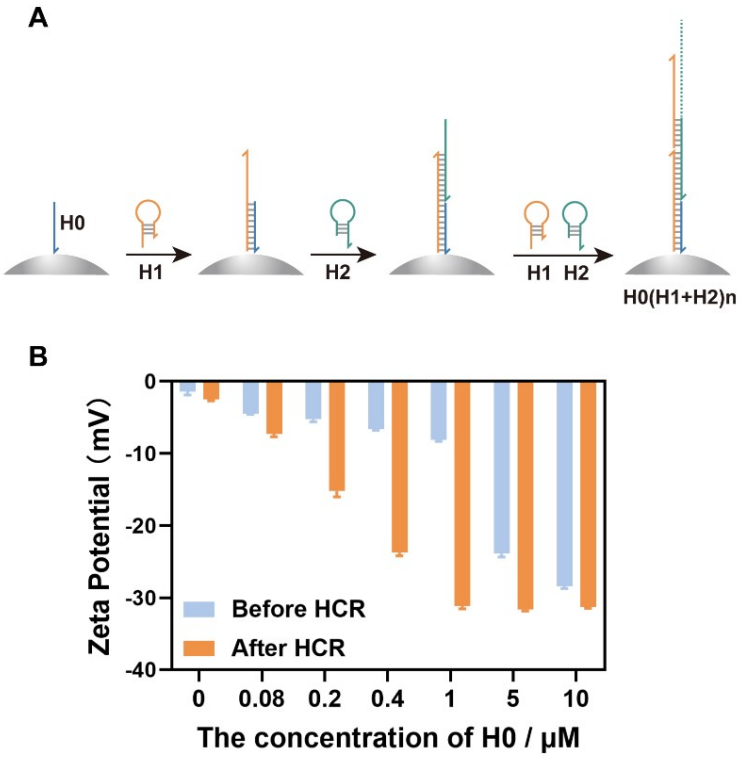

Figure S9. HCR among initiator MBs-H0, H1 and H2 on the MBs: (A) schematic illustration; and (B) changes in zeta potentials before and after HCR.

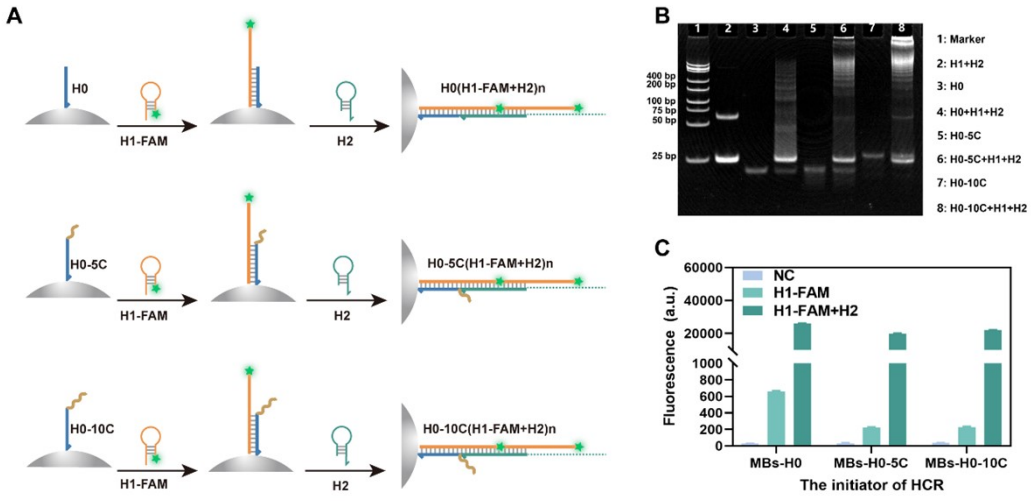

Figure S10. HCR among initiator MBs-H0 with different poly C tails, H1-FAM and H2 on the MBs: (A) schematic illustration; (B) polyacrylamide gel electrophoresis analysis of H0, H0-5C, H0-10C before and after HCR assembly with H1-FAM and H2 in solution; and (C) fluorescence intensities MBs-H0, MBs-H0-5C and MBs-H0-10C after stepwise assembly with H1-FAM and H2.

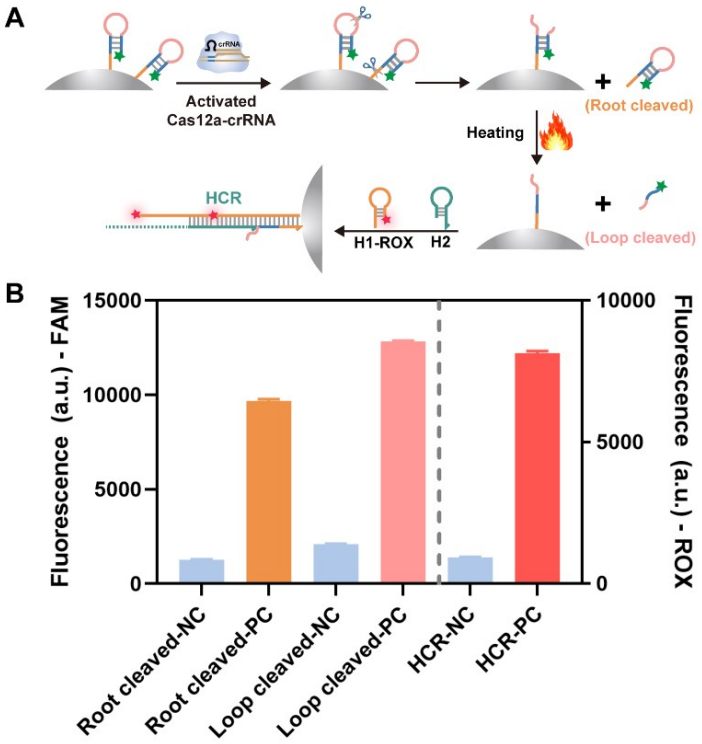

Figure S11. (A) Schematic illustration of CRISPR-Cas12a mediated *trans*-cleavage of FAM-hairpin DNA-MBs and subsequent HCR assembly with H1-ROX and H2 on the MBs, the root, stem and loop of the hairpin DNA were 10 nt, 14 bp and 10 nt in length, respectively; and (B) fluorescence intensities of the root-cleaved and loop-cleaved hairpin DNA-MBs, as well as the loop-cleaved hairpin DNA-MBs after heating denaturing and HCR assembly with H1-ROX and H2.

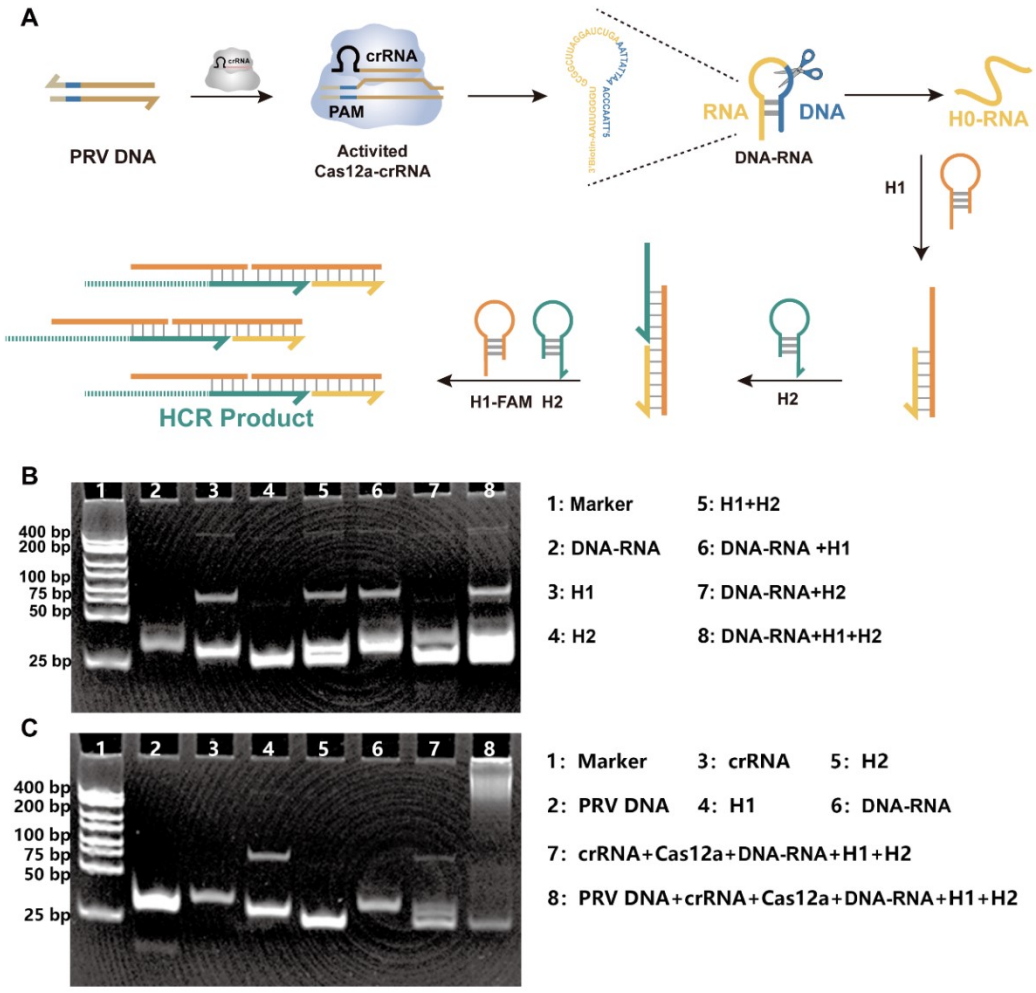

Figure S12. (A) Schematic illustration of PRV DNA-activated CRISPR-Cas12a *trans*-cleavage of DNA-RNA chimeric hairpins and subsequent HCR assembly with H1-FAM and H2; PAGE analysis of (B) the products from the HCR assembly among DNA-RNA chimeric hairpin, H1 and H2 and (C) the products from the HCR assembly among Cas12a-cleaved DNA-RNA chimeric hairpin, H1 and H2.

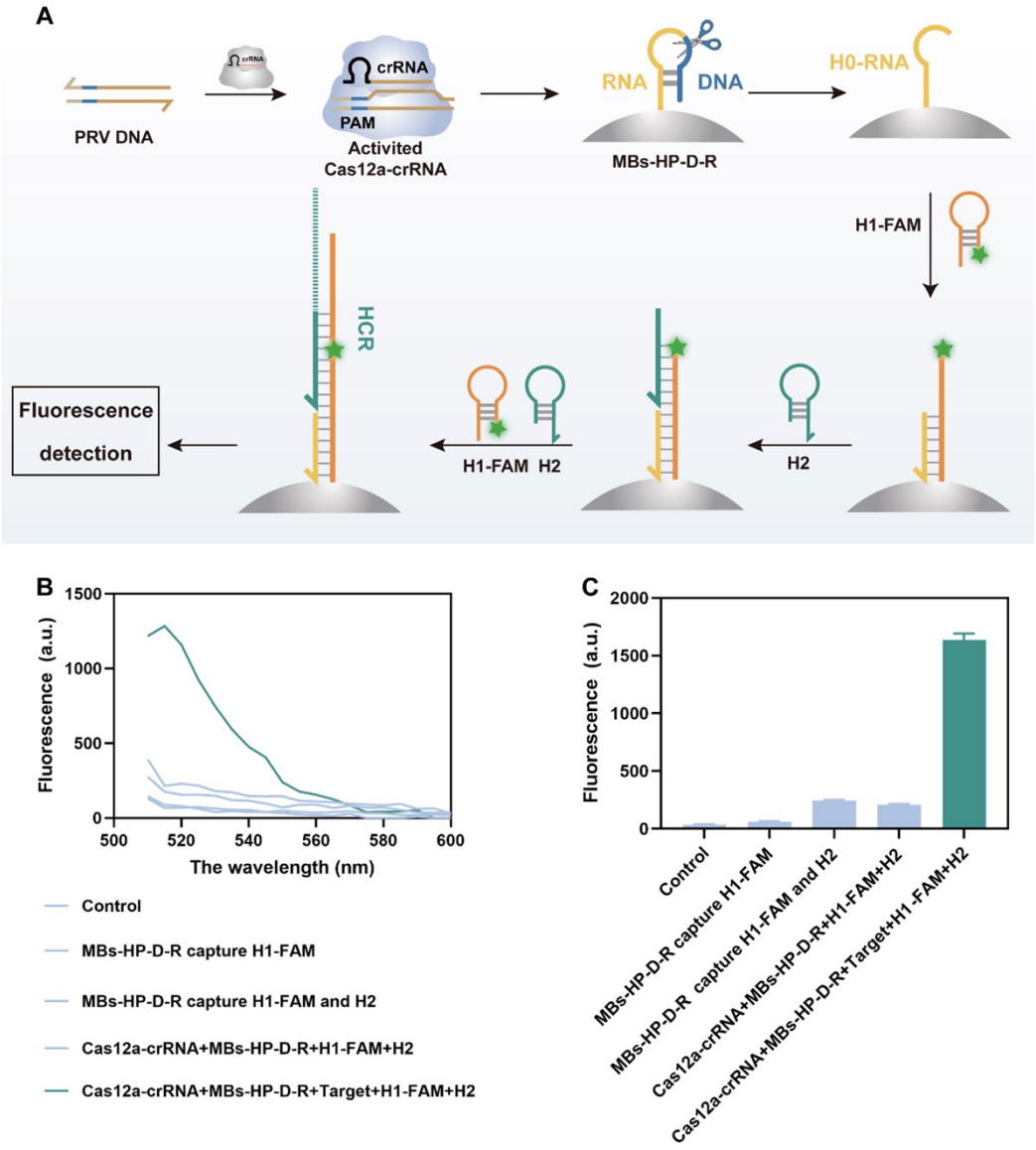

Figure S13. (A) Schematic illustration of PRV DNA-activated CRISPR-Cas12a *trans*-cleavage of MBs-HP-D-R and subsequent HCR assembly with H1-FAM and H2; and (B, C) fluorescence spectra and intensities from the HCR assembly among Cas12a-cleaved MBs-HP-D-R, H1 and H2.

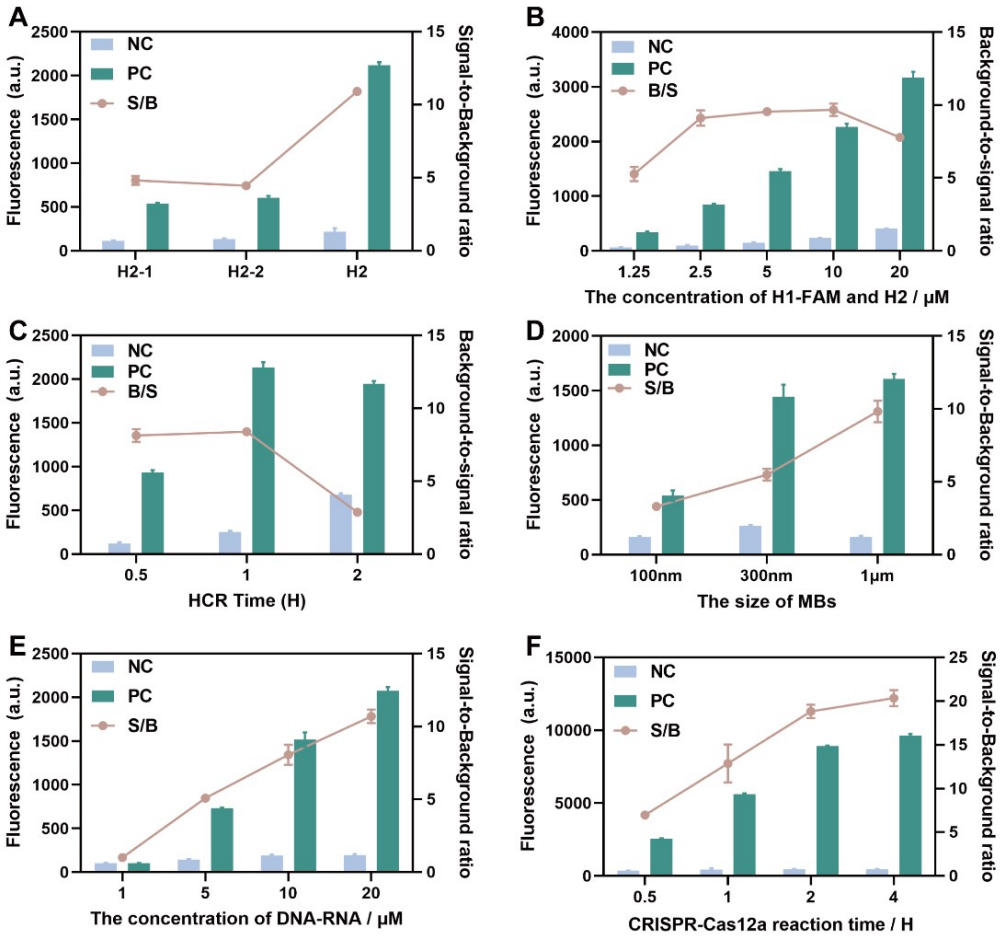

Figure S14. The impact of: (A) H2 sequence; (B) the concentration of H1-FAM and H2; (C) HCR time; (D) the size of MBs; (E) the concentration of DNA-RNA chimera hairpin and (F) the reaction time of CRISPR-Cas12a on the fluorescence intensities from the HCR assembly among Cas12a-cleaved MBs-HP-D-R, H1 and H2.

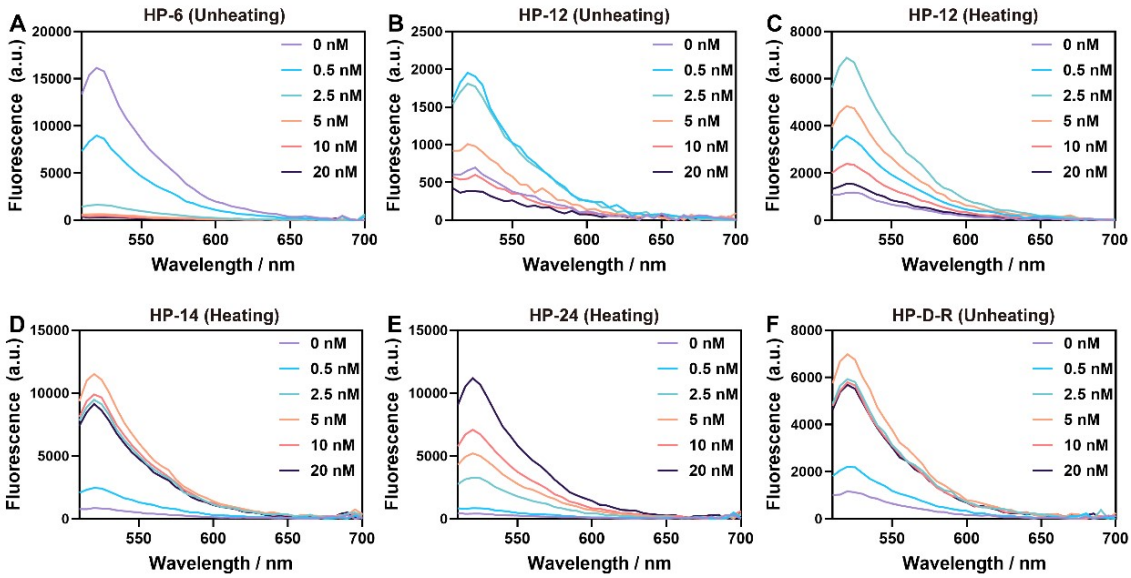

Figure S15. CRISPR-Cas12a-mediated detection of PRV DNA employing different MBs-HP as cleaving substrates and HCR for amplification. Fluorescence spectra for each PRV DNA concentration using: (A) MBs-HP-6 as trans-cleavage substrates without heating denaturing; (B) MBs-HP-12 as trans-cleavage substrates without heating denaturing; (C) MBs-HP-12 as trans-cleavage substrates with heating denaturing; (D) MBs-HP-14 as trans-cleavage substrates with heating denaturing; (E) MBs-HP-24 as trans-cleavage substrates with heating denaturing; or (F) MBs-HP-D-R as trans-cleavage substrates without heating denaturing.

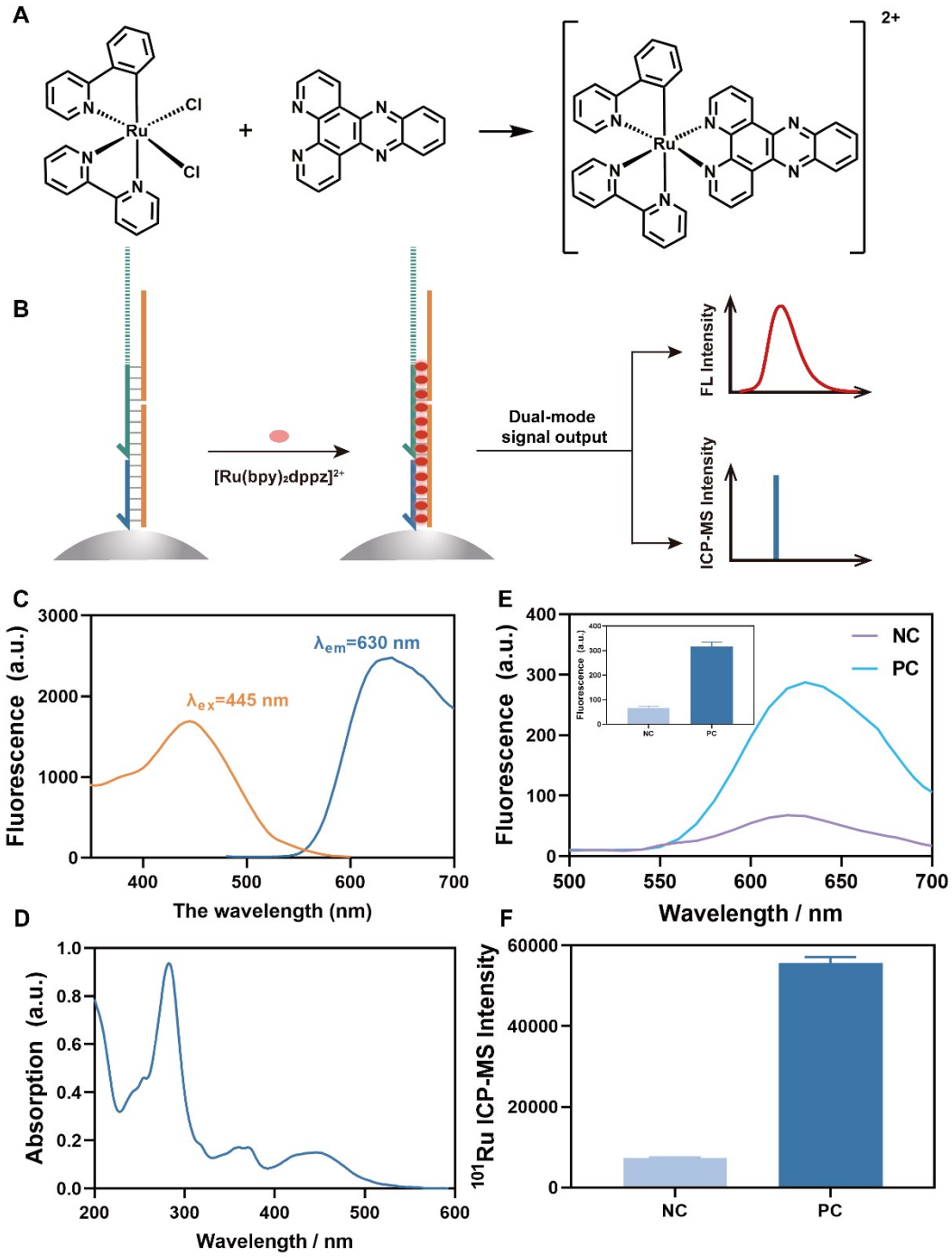

Figure S16. (A) Synthesis of Ru-dppz; (B) schematic illustration of Ru-dppz as intercalators for the dual-mode FL/ICP-MS detection of PRV DNA; (C) excitation and fluorescence emission spectra of Ru-dppz; (D) UV-visible absorption spectrum of Ru-dppz; (E) fluorescence spectra of Ru-dppz for the fluorescent detection of PRV DNA (inset were histograms of corresponding fluorescence intensities); and (F) feasibility of Ru-dppz for ICP-MS detection of PRV DNA.

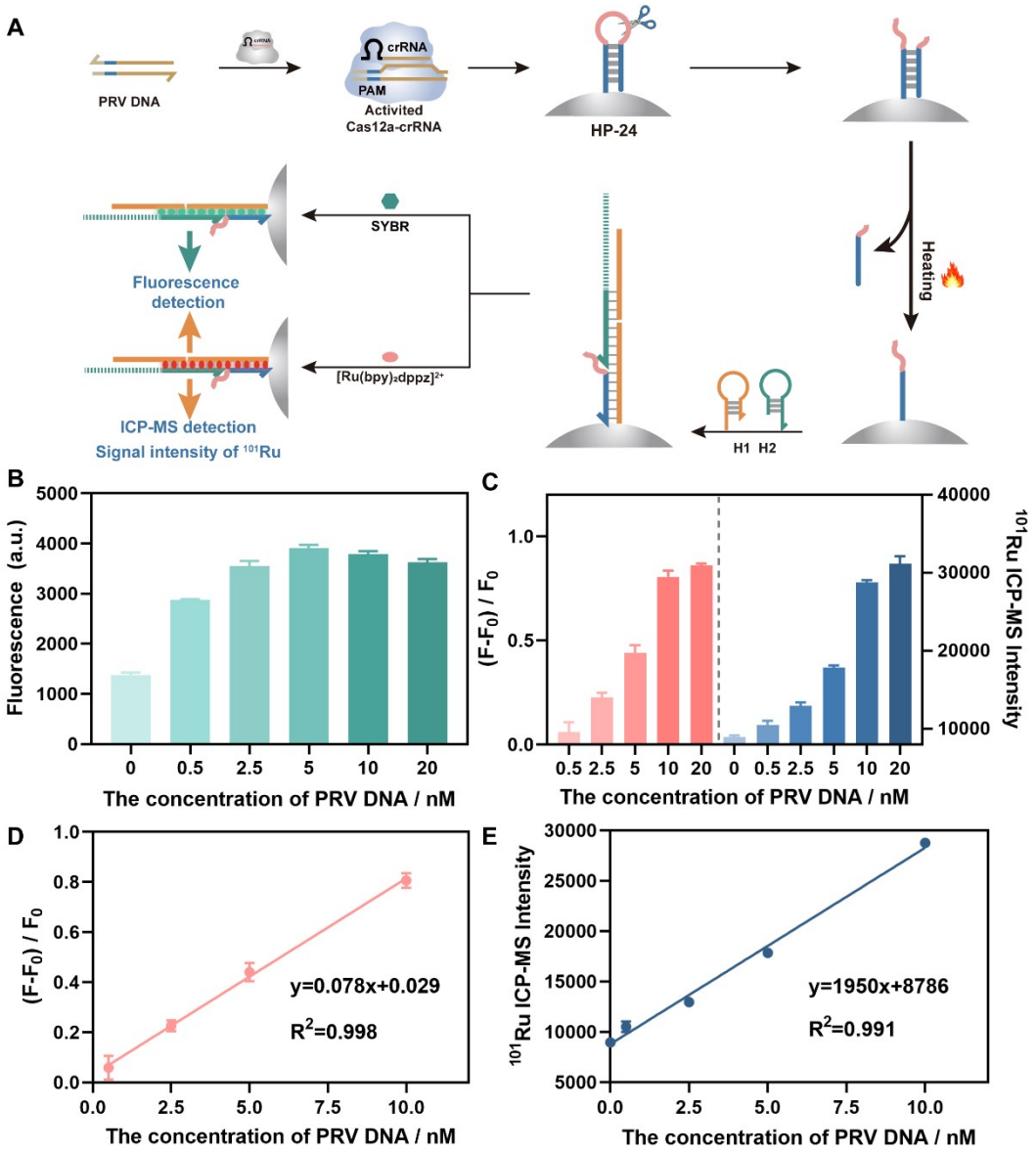

Figure S17. (A) Schematic illustration of PRV DNA-activated Cas12a *trans*-cleavage of MBs-HP-24 and HCR for the label-free detection PRV DNA using SYBR Green I or Ru-dppz as DNA intercalators; (B) the relationship between the fluorescence intensity of SYBR Green I and the concentration of PRV DNA; (C, D) the relationship between the fluorescence intensity of Ru-dppz and the concentration of PRV DNA; and (E) the relationship between the ICP-MS intensity of ^{101}Ru and the concentration of PRV DNA.

Table S4. Clinical diagnosis of possible PRV infections in swine using RT-qPCR and the dual-mode approach developed in this work.

Sample number	Ct value	Diagnosis by RT-qPCR	Diagnosis by the dual-mode approach
S1	>40	Negative	Negative
S2	>40	Negative	Negative
S3	>40	Negative	Negative
S4	>40	Negative	Negative
S5	>40	Negative	Negative
S6	>40	Negative	Negative
S7	15.10	Positive	Positive
S8	15.42	Positive	Positive
S9	14.69	Positive	Positive
S10	21.61	Positive	Positive
S11	23.18	Positive	Positive
S12	22.57	Positive	Positive

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