Supplementary Information

A Ligation-driven CRISPR-Cas Biosensing Platform for Non-Nucleic Acid Target Detections

Jiali Zhao[†a], Zhen Tan[†a], Liu Wang*[b], Chunyang Lei*[a] and Zhou Nie[a]

[a] State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan Provincial Key Laboratory of Biomacromolecular Chemical Biology, Hunan University, Changsha 410082, P. R. China. E-mail: cylei@hnu.edu.cn.

[b] State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-products; Institute of Agro-product Safety and Nutrition, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China. Email: wangliually@126.com.

[†] These authors contributed equally to this work.

[*] Corresponding author.

Contents

Experimental section

Materials and reagents. T4 DNA ligase, T4 Polynucleotide Kinase and ATP were purchased from Thermo Scientific (Thermo Scientific, Waltham, MA). *E.coli* DNA ligase was purchased from Takara Bio. β-Nicotinamide adenine dinucleotide (NAD^+) was purchased from Sigma Aldrich (St. Louis, MO, USA). Reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Solarbio (Beijing, China). Uridine triphosphate (UTP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and guanosine triphosphate (GTP) were purchased from Sangon Biotech (Shanghai, China) and all other chemical reagents were reached to analytical reagent grade and used without further purification. The solutions were prepared with ultrapure water (18.25 MΩ cm, Millipore Milli-Q system), and all solutions containing RNA were prepared with ultrapure water treated with diethyl pyrocarbonate (DEPC). All DNA oligos were synthesized and purified by Sangon Biotech (Shanghai, China). The real-time fluorescent quantitative PCR instrument (QuantStudio 7 Flex) used in the experiment was purchased from Applied Biosystems (Biosystems/Ambion, Foster City, CA, USA).

Protein expression and purification. The pET28a-LbCas12a plasmid was transformed into Escherichia coli Rosetta (DE3). When expressing the protein, a monoclonal colony was picked from solid LB medium and cultured overnight in 6 mL liquid LB medium containing Kana antibiotics (37 ºC, 250 rpm). The obtained culture was added to the liquid LB medium in a volume ratio of 1:100 to expand the culture. When the E. coli were in the logarithmic phase, the inducer IPTG with a final concentration of 0.2 mM was added to the culture to induce the expression of LbCas12a protein (16 ºC, 160 rpm, 16 h). After the induction of expression, the bacterial cells were lysed by ultrasonic in lysis buffer (50 mM Tris-HCl, 1.5 M NaCl, pH 8.0), and the supernatant was collected by centrifugation, then filtered supernatant was added to the Ni-NTA affinity column. The packing was then washed with wash buffer (lysis buffer supplemented with 30 mM imidazole, pH 8.0) and eluted with elution buffer (lysis buffer supplemented with 600 mM imidazole, pH 8.0). Because the eluted protein solution contains high concentration of imidazole and inorganic salts, which is not conducive to the storage of LbCas12a and may affect subsequent experiments, we used HiTrap™ desalting column to replace LbCas12a protein into storage buffer (20 mM Tris-HCl, 600 mM NaCl, 2 mM DTT, 10% glycerol, pH 8.0) and then stored at -80 ºC. The SDS-PAGE image of LbCas12a protein solution obtained by purification and desalting is exhibited in Fig. S1.

The detection of NAD^+ and ATP. The three strands ([DNA1]:[DNA2]:[NTS] = 1:1:1.5) were hybridized to get gapped dsDNA. In a typical DNA ligation reaction, 10 μL of ligase reaction buffer solution (60 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.5) contains the following components: 500 nM gapped dsDNA, 3 U E.coli DNA ligase or 1 U T4 DNA ligase, and different concentrations of NAD^+ or ATP. The mixture was incubated at 37 ºC for 30 min for ligation, then heated at 90 ºC for 5 min to inactivate the DNA ligase, and cooled to room temperature naturally. Then pre-assembled LbCas12a-crRNA, ssDNA reporter and CRISPR reaction buffer were added to the reaction solution for the Cas12a trans-cleavage reaction. The final 20 μL CRISPR reaction buffer solution (20 mM Tris-HCl, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 50 μg/mL heparin, and pH 7.5) contains the following components: 50 nM LbCas12a-crRNA, 1 μM ssDNA reporter and the product of the above ligation reaction (50 nM dsDNA). The reaction was carried out in a real-time fluorescent quantitative PCR instrument at 37 ºC, and fluorescence measurements were taken every 30 seconds.

The detection of T4 PNK activity. In a typical T4 PNK-mediated phosphorylation reaction, 5 μL of PNK reaction buffer solution (50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, pH 8.0) contains 1 μM DNA1-OH,

1mM ATP and different concentrations of T4 PNK, the reaction was carried out at 35 ºC for 45 min. Then the three strands hybridized (The hybrid ratio was the same as above) to get nicked dsDNA.

Then the DNA ligation reaction was performed, 10 μL of ligase reaction buffer solution contains the following components: 500 nM gapped dsDNA, 1 U T4 DNA ligase and 1 mM ATP, the reaction was incubated at 37 ºC for 30 min, then heated at 90 ºC for 5 min to inactivate the DNA ligase, and naturally cooled to room temperature. Next, the Cas12a trans-cleavage reaction was performed, and the experimental steps were the same as above.

T4 PNK inhibition assay. In order to study whether this method can be used to evaluate and screen T4 PNK inhibitors, we used ammonium sulfate [$(NH_4)_2SO_4$] for the inhibition experiment. The experimental procedure was similar to the activity determination of T4 PNK, except that different concentrations of inhibitors were added to the phosphorylation reaction buffer solution in the first step, and the concentration of T4 PNK was fixed at 1 U/mL.

Detection of endogenous PNK activity in Hela cells: HeLa cells were grown to the logarithmic growth phase in DMEM medium at 37 ºC under 5% CO_2. About 5×10^6 cells were washed twice with PBS and then lysed. The cell lysates were obtained by using the Nucleoprotein Extraction Kit (Sangon Biotech, Shanghai, China, C500009), and the extraction procedures were consistent with the instructions. The lysis products were stored at -80 ºC or used immediately. The following detection operations were similar to the activity determination of T4 PNK, except that cell lysates were added in the sensing system instead of T4 PNK. In order to verify that the fluorescent signal generated by the lysates were due to the PNK activity rather than other components, the lysis buffer, cell lysates (6000 cells), heat-inactivated cell lysates (treated at 95 ºC for 10 min) and cell lysates treated with inhibitor $(NH_4)_2SO_4$ (30 mM) were tested separately. Then the signal responses of the sensing system caused by different concentrations of Hela cell lysates (100, 200, 500, 1000, 1500, 3000, 4500, 6000 cells) were measured.

Fluorescence measurements. The Cas12a-mediated nucleic acid hydrolysis reaction and fluorescence intensity measurement were performed on a real-time fluorescent quantitative PCR instrument (QuantStudio 7 Flex). The excitation wavelength of the fluorescent group 5-carboxyfluorescein (5-FAM) was set at 488 nm, and the emission at 520 nm were recorded. The volume of each sample in the 96-well plate was 20 μL, and the fluorescence intensity was measured every 30 s at 37 ºC.

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

Names	sequences（5'-3'）
TS	TCATAGTTAGCGTAACGATCTAAAGTTTTGTCGTC[a,b]
DNA1	TAACGATCTAAAGTTTTGTCGTC
DNA2	TCATAGTTAGCG
DNA1-0	TAAAGTTTTGTCGTC
DNA2-0	TCATAGTTAGCGTAACGATC
DNA1-2	TCTAAAGTTTTGTCGTC
DNA2-2	TCATAGTTAGCGTAACGA
DNA1-PO_4	PO_4-TAACGATCTAAAGTTTTGTCGTC
DNA1-OH	HO-TAACGATCTAAAGTTTTGTCGTC
NTS	GACGACAAAACTTTAGATCGTTACGCTAACTATGA[c]
ssDNA reporter	FAM-TTATT-BHQ1
crRNA	UAAUUUCUACUAAGUGUAGAUGAUCGUUACGCUAACUAUGA

[a] The bases with underline and red color are the complementary sequence of PAM.

[b] The bases with blue color are the sequence targeted by the spacer region of crRNA.

[c] The bases with red color are the sequence of PAM.

Table S2 Comparison of our PNK sensing system with other reported methods.

Method	LOD (U/mL)	Sample	Ref
Ligation-triggered DNAzyme cascade amplification	0.001	PNK spiked serum	1
Label-free G-quadruplex-based luminescent assay	0.05	PNK spiked cell lysates	2
Allosteric aptamer probe platform	0.01	PNK spiked cell lysates	3
Dual-enzyme-assisted three-dimensional DNA walker	0.0067	PNK spiked serum	4
GQD-CuNC nanohybrid-based ratio fluorescence approach	0.0037	PNK spiked cell lysates	5
Magnetic bead-AuNPs hybrids based dark field microscope	0.0058	PNK spiked cell lysates	6
Ligation-driven CRISPR-Cas biosensing platform	0.0005	Endogenic PNK in cell lysates	This work

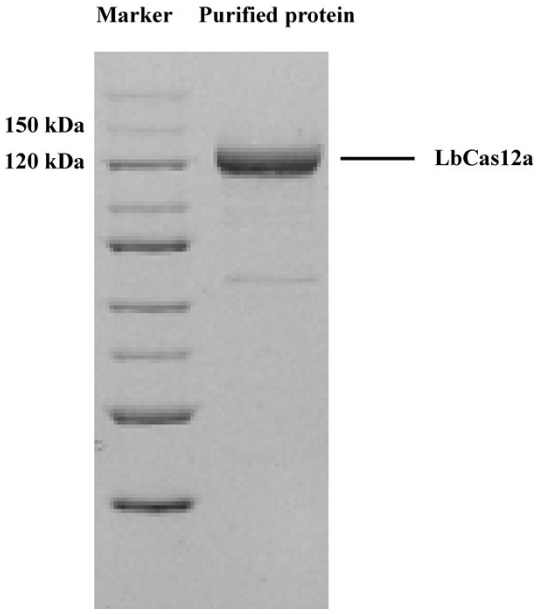

Fig. S1 SDS-PAGE image of the purified LbCas12a protein.

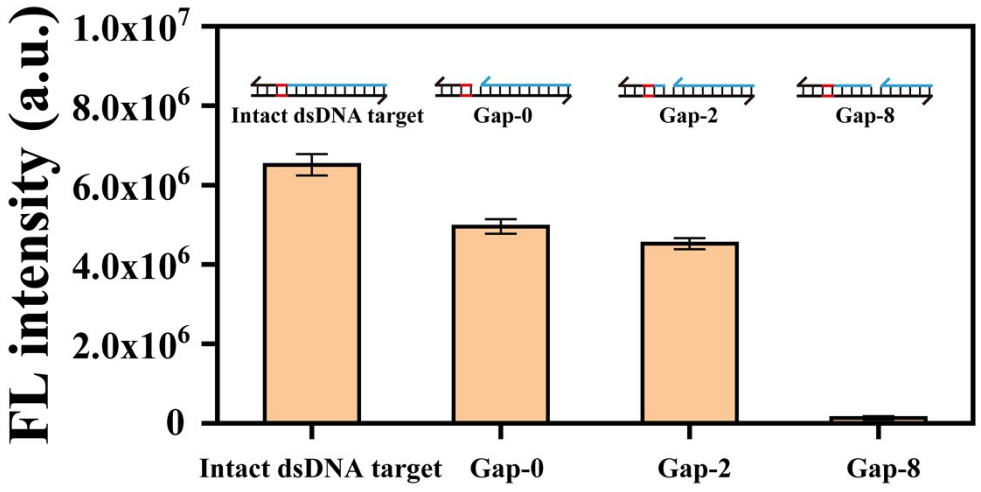

Fig. S2 Fluorescence signals of LbCas12a's *trans*-cleavage activated by different gapped dsDNA. Gap-0, Gap-2 and Gap-8 are the dsDNA targets containing a gap at the position 0, +2 and +8 in TS, respectively. The concentration of each dsDNA target, 50 nM; the concentration of LbCas12a-crRNA, 50 nM; the concentration of ssDNA reporter, 1 μM.

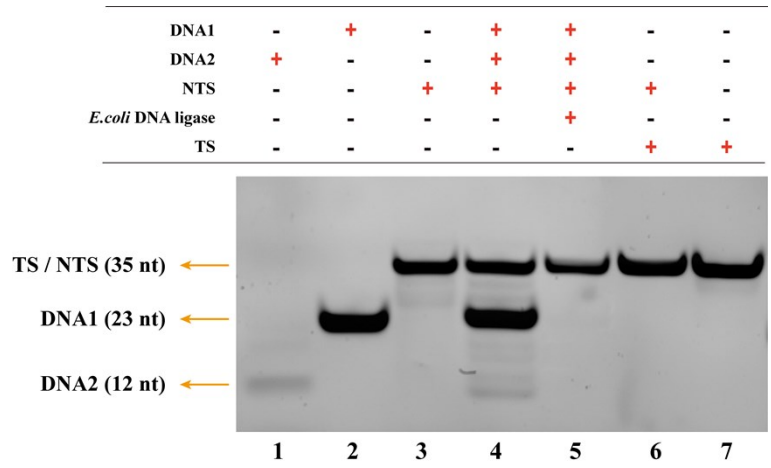

Fig. S3 Denaturing PAGE (15%) analysis of the ligation products generated by the DNA ligation reaction. Lane 1: DNA2, 360 pmol; lane 2: DNA1, 15 pmol; lane 3: NTS, 17 pmol; lane 4: DNA2 (12 pmol) + DNA1 (12 pmol) + NTS (12 pmol); lane 5: ligation products of DNA2 (5 pmol) + DNA1 (5 pmol) + NTS (5 pmol); lane 6: duplex of NTS (12 pmol) and TS (12 pmol); and lane 7: TS, 26 pmol. In lane 5, the disappearance of the bands of NDA1 and DNA2 demonstrate that DNA ligase can connect the gap to obtain intact dsDNA targets.

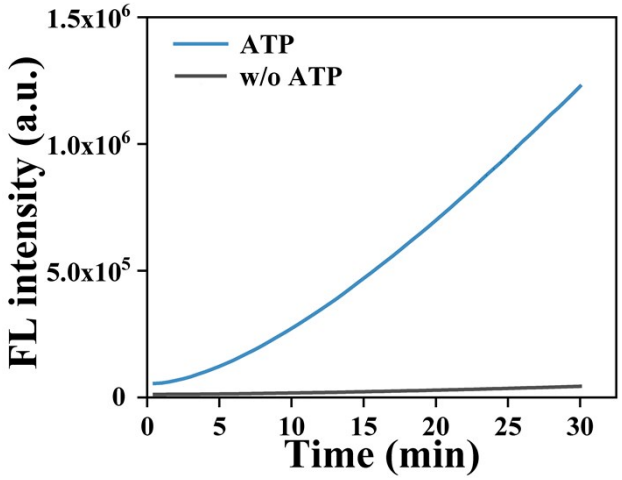

Fig. S4 The fluorescence response before and after adding 0.5 μM ATP to the system containing T4 DNA ligase. The concentration of DNA, 50 nM; the concentration of T4 DNA ligase, 0.1 U/μL; the concentration of LbCas12a-crRNA, 50 nM; the concentration of ssDNA reporter, 1 μM.

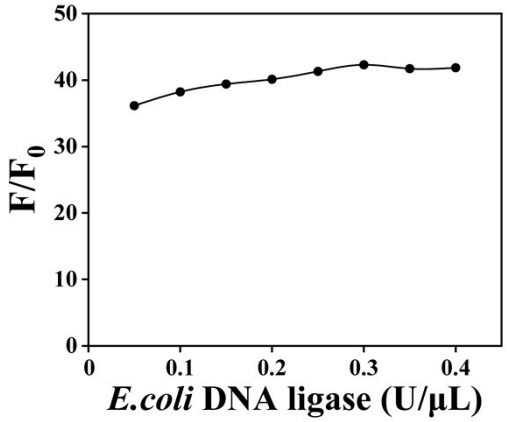

Fig. S5 Optimization of *E.coli* DNA ligase concentration in NAD^+ assay. The final concentration of DNA, 50 nM; the concentration of NAD^+, 1 μM; the concentration of LbCas12a-crRNA, 50 nM; the concentration of ssDNA reporter, 1 μM. The concentration of *E.coli* DNA ligase was optimized in the range from 0.05 to 0.4 U/μL, and the ratio of signal-to-background reached saturation at 0.3 U/μL of *E.coli* DNA ligase. The final determined *E.coli* DNA ligase concentration, 0.3 U/μL.

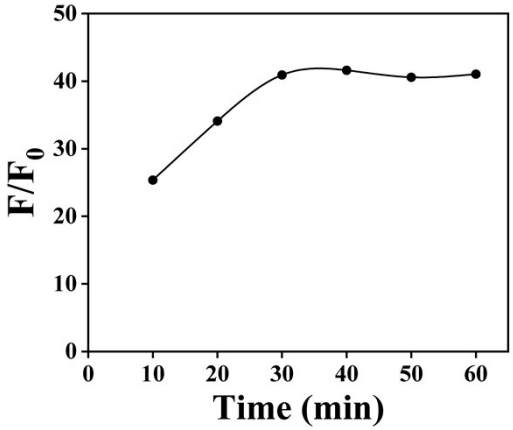

Fig. S6 Optimization of ligation reaction time in NAD^+ assay. The concentration of DNA, 50 nM; the concentration of NAD^+, 1 μM; the concentration of *E.coli* DNA ligase, 0.3 U/μL; the concentration of LbCas12a-crRNA, 50 nM; the concentration of ssDNA reporter, 1 μM. The ligation reaction time in NAD^+ assay was optimized in the range from 10 to 60 min, and the ratio of signal-to-background reached saturation at the ligation reaction time of 30 min. The final determined ligation reaction time was 30 min.

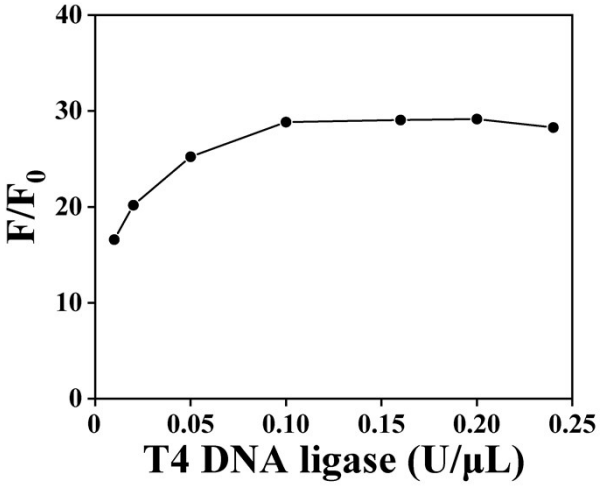

Fig. S7 Optimization of T4 DNA ligase concentration in ATP assay. The concentration of DNA, 50 nM; the concentration of ATP, 1 μM; the concentration of LbCas12a-crRNA, 50 nM; the concentration of ssDNA reporter, 1 μM. The concentration of T4 DNA ligase was optimized in the range from 0.01 to 0.24 U/μL, and the ratio of signal-to-background reached saturation at 0.1 U/μL of T4 DNA ligase. The final determined T4 DNA ligase concentration, 0.1 U/μL.

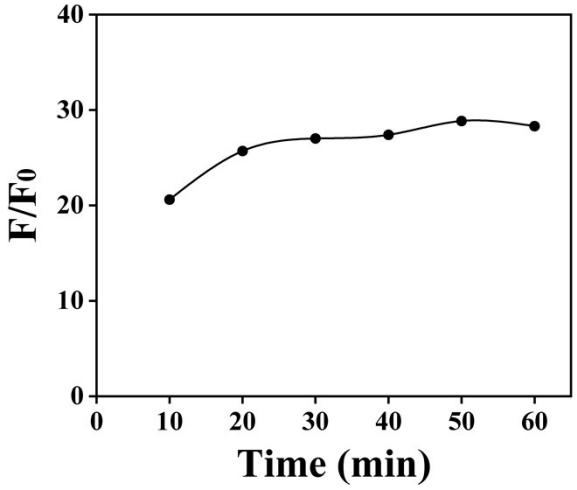

Fig. S8 Optimization of ligation reaction time in ATP assay. The concentration of DNA, 50 nM; the concentration of ATP, 1 μM; the concentration of T4 DNA ligase, 0.1 U/μL; the concentration of LbCas12a-crRNA, 50 nM; the concentration of ssDNA reporter, 1 μM. The ligation reaction time in ATP assay was optimized in the range from 10 to 60 min, and the ratio of signal-to-background basically reached saturation at the ligation reaction time of 30 min. The final determined ligation reaction time was 30 min.

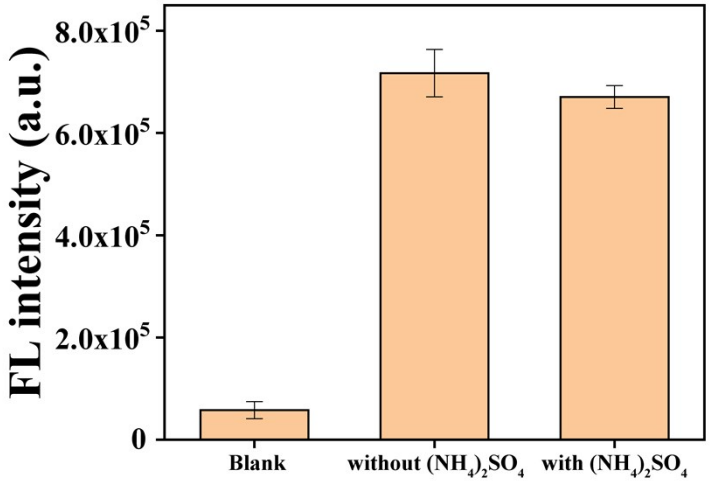

Fig. S9 Effect of $(NH_4)_2SO_4$ on the T4 DNA ligase/Cas12a system. DNA1-OH was treated with T4 PNK firstly, and the phosphorylated product hybridized with DNA2 and NTS to obtain gapped dsDNA. The inhibitor $(NH_4)_2SO_4$ (25 mM) was added to the reaction substrate (gapped dsDNA), and then T4 DNA ligase and Cas12a were added successively for ligation reaction and Cas12a-mediated nucleic acid hydrolysis reaction. The concentration of T4 PNK, 5 U/mL; the concentration of gapped dsDNA, 50 nM; the concentration of T4 DNA ligase, 0.1 U/μL; the concentration of LbCas12a-crRNA, 50 nM; the concentration of ssDNA reporter, 1 μM.

References

1. S. F. Liu, J. J. Ming, Y. Lin, C. F. Wang, C. B. Cheng, T. Liu and L. Wang, Biosens Bioelectron, 2014, **55**, 225-230.
2. H. Z. He, K. H. Leung, W. Wang, D. S. H. Chan, C. H. Leung and D. L. Ma, Chem Commun, 2014, **50**, 5313-5315.
3. M. X. Gao, J. J. Guo, Y. L. Song, Z. Zhu and C. J. Yang, Acs Appl Mater Inter, 2017, **9**, 38356-38363.
4. C. Feng, Z. H. Wang, T. S. Chen, X. X. Chen, D. S. Mao, J. Zhao and G. X. Li, Anal Chem, 2018, **90**, 4932-4932.
5. M. K. Wang, D. S. Kong, D. D. Su, Y. Liu and X. G. Su, Nanoscale, 2019, **11**, 13903-13908.
6. T. Jin, J. W. Zhang, Y. F. Zhao, X. T. Huang, C. Y. Tan, S. Q. Sun and Y. Tan, Biosens Bioelectron, 2020, **150**, 111936.