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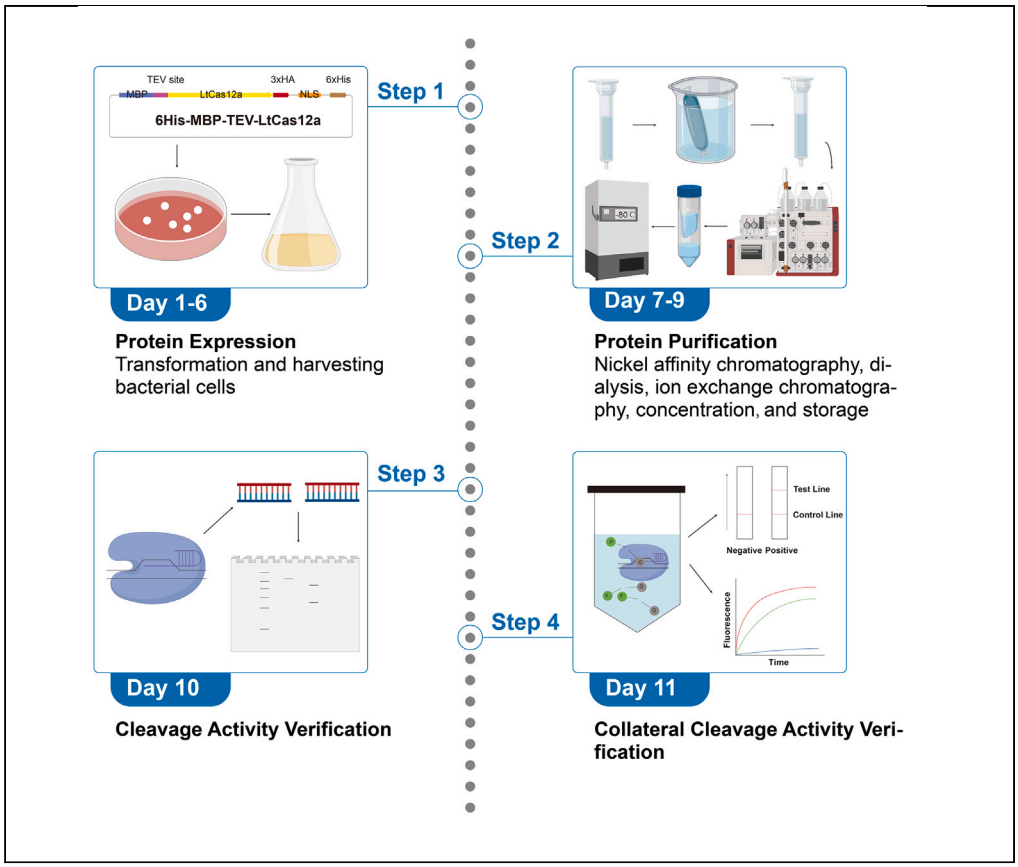
Protocol

Purification and functional validation of LtCas12a protein



Here, we present a protocol for generating LtCas12a protein recognizing distinct TTNA (N represented A, T, C, G) protospacer adjacent motif sequence. We describe steps for transforming and harvesting bacterial cells and protein purification including nickel affinity chromatography and dialysis. We then detail procedures for verification of LtCas12a with cis- and trans-cleavage activities.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Bo Zhou, Ye Chen, Lifang Li, ..., Zheng Hu, Rui Tian, Zhen Li

huzheng1998@163.com (Z.H.)
tianruiaiya@gmail.com (R.T.)
lizhen9@gmail.com (Z.L.)

Highlights

Detailed protocol for a Cas12a ortholog with TTNA PAM

Purification of LtCas12a via multiple chromatographic techniques

Validation of LtCas12a biological characteristics by various *in vitro* assays

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Purification and functional validation of LtCas12a protein

Bo Zhou,[1,4,5] Ye Chen,[2,5,6] Lifang Li,[2,5] Jiashuo Liu,[2] Yuyan Wang,[2] Zheying Huang,[2] Zheng Hu,[1,4,7,*] Rui Tian,[3,*] and Zhen Li[1,4,*]

[1]Department of Gynecological Oncology, Zhongnan Hospital of Wuhan University, Wuhan 430062, Hubei, China

[2]Department of Gynecological Oncology, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, Guangdong, China

[3]Generulor Company Bio-X Lab, Zhuhai 519000, Guangdong, China

[4]Women and Children's Hospital Affiliated to Zhongnan Hospital of Wuhan University, Wuhan 430062, Hubei, China

[5]These authors contributed equally

[6]Technical contact: cy0914@163.com

[7]Lead contact

*Correspondence: huzheng1998@163.com (Z.H.), tianruiaiya@gmail.com (R.T.), lizhen9@gmail.com (Z.L.)
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SUMMARY

Here, we present a protocol for generating LtCas12a protein recognizing distinct TTNA (N represented A, T, C, G) protospacer adjacent motif sequence. We describe steps for transforming and harvesting bacterial cells and protein purification including nickel affinity chromatography and dialysis. We then detail procedures for verification of LtCas12a with cis- and trans-cleavage activities.

For complete details on the use and execution of this protocol, please refer to Chen et al. (2023).[1]

BEFORE YOU BEGIN

This protocol was applied in a recent publication (Chen et al., 2023)[1] to generate and purify LtCas12a nuclease in *Escherichia coli* (*E. coli*). Before the experiment, expression vector selection and physicochemical prediction of LtCas12a are recommended for protein expression and production.

Construct preparation

Timing: 3 days

1. Clone LtCas12a codon-optimized sequence in the bacterial expression vector 6His-MBP-TEV-huAsCpf1 vector[2] (Addgene, #90095) containing an N-terminal 6xHis-maltose binding protein (6xHis-MBP) tag.

Parameter preparation

Timing: 1 h

2. Obtain various physical and chemical parameters for LtCas12a in **ExPASy Server**[3] (the Expert Protein Analysis System) (https://web.expasy.org/protparam/).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Trans5α Chemically Competent Cell	Trans	Cat#CD201-01
BL21(DE3) Chemically Competent Cell	Trans	Cat#CD601-02
Chemicals, peptides, and recombinant proteins		
2xMultiF Seamless Assembly Mix	ABclonal	Cat#RK21020
2xPhanta Max Master Mix	Vazyme	Cat#P515-01
Ampicillin sodium salt	Macklin	Cat# A6265
Isopropyl-b-D-thiogalactopyranoside (IPTG) Solution	Sangon	Cat#B541007-0001
Sodium chloride	Sangon	Cat#A610476
Glutathione reduced	Sangon	Cat#A600229
HEPES	Macklin	Cat#H6282
Ni Sepharose 6 FF	Cytiva	Cat# 17531801
2-Mercaptoethanol (β-ME)	Sigma	Cat# M3148
Triton-X 100	Macklin	CAS# 9002-93-1
Imidazole	Sigma	Cat# I5513
Glycerol	Sangon	Cat# A100854
Tris	Macklin	Cat# R854237
DTT	Aladdin	Cat# D104860
EDTA	Sigma	Cat# EDS-100G
Magnesium chloride hexahydrate	Sangon	Cat#A601336
Phenylmethylsulfonyl fluoride (PMSF)	Sigma	Cat#P8340
Molecular sieve	Sigma	Cat#208620
Mag-Beads	Sangon	Cat#C650031
NEBuffer 2.1	NEB	Cat#B7202V
Critical commercial assays		
SDS-PAGE Gel Preparation Kit	Yeasen	Cat#20328ES50
HiScribe T7 High Yield RNA Synthesis Kit	NEB	Cat#E2040S
Monarch RNA Cleanup Kit	NEB	Cat#T2040L
EasyPure® Quick Gel Extraction Kit	Trans	Cat#EG101-01
Lateral flow strip kit	Milenia	Cat#MGHD 1
Deposited data		
Amino acid sequence of LtCas12a	This paper-Table S1	N/A
Target sequences	This paper-Table S2	N/A
Primer sequences	This paper-Table S3	N/A
Oligonucleotides		
5′-FAM-ssDNA (TTATT)-BHQ-3′ reporter	Sangon	N/A
5′-FAM-ssDNA (TTATT)- Biotin -3′ reporter	Sangon	N/A
Recombinant DNA		
6His-MBP-TEV-huAsCpf1	Addgene	Cat#90095
Other		
Avanti Ultracentrifuge	Beckman	Cat#JXN-26
Ultra-high pressure continuous flow cell crusher	JNBIO	Cat#JN-2.5C
Ultrasonic cell pulverizer	Scientz	Cat#JY92-IIN
Dialysis bag	Asegene	Cat#as77500
0.45 μm filter paper	PALL	Cat#60173
0.45 μm MILLEX-HV syringe filter	Merck millipore	Cat#SLHVR33RB
Gravity flow column	IBA	Cat#2-5012-001
Fast protein liquid chromatography, FPLC	Inscinstech	Cat#Unique AutoPure100
SP Sepharose High Performance	Cytiva	Cat#17108704
Centrifuge concentrator	Merck	Cat#UFC710008
LineGene 9600 Plus real time PCR detection system	BIOER	Cat#FQD-96A

MATERIALS AND EQUIPMENT

Resuspension buffer

Reagent	Final concentration	Amount
Tris (1M, pH 7.5, 25°C)	40 mM	40 mL
NaCl	500 mM	29.22 g
Imidazole (3 M)	20 mM	6.67 mL
Glycerol	5%	50 mL
ddH_2O	N/A	Up to 1L
Total	**N/A**	**1L**

Resuspension buffer can be stored at 4°C for up to 6 months after preparation.

Equilibration buffer

Reagent	Final concentration	Amount
Tris (1 M, pH 7.5, 25°C)	40 mM	40 mL
NaCl	500 mM	29.22 g
Imidazole (3 M)	20 mM	6.67 mL
Glycerol	5%	50 mL
2-Mercaptoethanol (14.3 M)	2 mM	139.86 µL
ddH_2O	N/A	Up to 1 L
Total	**N/A**	**1 L**

Equilibration buffer can be stored at 4°C for up to 6 months after preparation.

Washing buffer 1

Reagent	Final concentration	Amount
Tris (1 M, pH 7.5, 25°C)	40 mM	40 mL
NaCl	500 mM	29.22 g
Imidazole (3 M)	20 mM	6.67 mL
Glycerol	5%	50 mL
2-Mercaptoethanol (14.3 M)	2 mM	139.86 µL
ddH_2O	N/A	Up to 1 L
Total	**N/A**	**1 L**

Washing buffer 1 can be stored at 4°C for up to 6 months after preparation.

Elution buffer 1

Reagent	Final concentration	Amount
Tris (1 M, pH 7.5, 25°C)	40 mM	40 mL
NaCl	500 mM	29.22 g
Imidazole (3 M)	300 mM	100 mL
Glycerol	5%	50 mL
2-Mercaptoethanol (14.3 M)	2 mM	139.86 µL
ddH_2O	N/A	Up to 1 L
Total	**N/A**	**1 L**

Elution buffer 1 can be stored at 4°C for up to 6 months after preparation.

Dialysis buffer

Reagent	Final concentration	Amount
Tris (1 M, pH 7.5, 25°C)	20 mM	20 mL
NaCl	250 mM	14.61 g
Glycerol	5%	50 mL
2-Mercaptoethanol (14.3M)	2 mM	139.86 µL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
ddH_2O	N/A	Up to 1 L
Total	**N/A**	**1 L**

Dialysis buffer can be stored at 4°C for up to 6 months after preparation.

Washing buffer 2		
Reagent	Final concentration	Amount
Tris (1 M, pH 7.5, 25°C)	20 mM	20 mL
NaCl	250 mM	14.61 g
Imidazole (3 M)	20 mM	6.67 mL
Glycerol	5%	50 mL
2-Mercaptoethanol (14.3 M)	2 mM	139.86 µL
ddH_2O	N/A	Up to 1 L
Total	**N/A**	**1 L**

Washing buffer 2 can be stored at 4°C for up to 6 months after preparation.

Elution buffer 2		
Reagent	Final concentration	Amount
Tris (1 M, pH 7.5, 25°C)	20 mM	20 mL
NaCl	250 mM	14.61 g
Imidazole (3 M)	500 mM	166.67 mL
Glycerol	5%	50 mL
2-Mercaptoethanol (14.3 M)	2 mM	139.86 µL
ddH_2O	N/A	Up to 1 L
Total	**N/A**	**1 L**

Elution buffer 2 can be stored at 4°C for up to 6 months after preparation.

Buffer A		
Reagent	Final concentration	Amount
Tris (1 M, pH 7.5, 25°C)	20 mM	20 mL
NaCl	250 mM	14.61 g
Glycerol	5%	50 mL
ddH_2O	N/A	Up to 1 L
Total	**N/A**	**1 L**

Buffer A can be stored at 4°C for up to 6 months after preparation.

Buffer B		
Reagent	Final concentration	Amount
Tris (1 M, pH 7.5, 25°C)	20 mM	20 mL
NaCl	1 M	58.44 g
Glycerol	5%	50 mL
ddH_2O	N/A	Up to 1 L
Total	**N/A**	**1 L**

Buffer B can be stored at 4°C for up to 6 months after preparation.

Size exclusion chromatography (SEC) buffer		
Reagent	Final concentration	Amount
Tris (1 M, pH 7.5, 25°C)	20 mM	20 mL
NaCl	250 mM	14.61 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
2-Mercaptoethanol (14.3 M)	2 mM	139.86 μL
ddH_2O	N/A	Up to 1 L
Total	**N/A**	**1 L**

SEC buffer can be stored at 4°C for up to 6 months after preparation.

Storage buffer		
Reagent	Final concentration	Amount
Tris (1 M, pH 7.5, 25°C)	20 mM	2 mL
NaCl	250 mM	1.461 g
Glycerol	5%	5 mL
ddH_2O	N/A	Up to 100 mL
Total	**N/A**	**100 mL**

Storage buffer can be stored at 4°C for up to 6 months after preparation.

STEP-BY-STEP METHOD DETAILS

Here, we provide a detailed step-by-step protocol for LtCas12a protein expression, purification, and identification of cleavage activity.

LtCas12a protein expression

Timing: 3 days

Expression of LtCas12a was conducted using competent *E. coli* BL21 (DE3) cells in 4 key steps listed below.

1. Transformation of Plasmids.
   a. Transform 6His-MBP-LtCas12a plasmids (200–300 ng/μL) into competent *Escherichia coli* BL21(DE3) (Transgen, China) with a volume ratio of 1:50.
   b. Add 500 μL antibiotic-free Lysogeny Broth (LB) medium into the competent *E. coli* cells and incubate them on a shaker at 37 °C at 200 rpm for 1 h.
   c. Plate 100–200 μL bacterial solution onto an LB agar plate containing ampicillin.
   d. Incubate the agar plate for 12 h at 37°C.

   Note: Using antibiotic-free LB medium for *E. coli* recovery just after transformation for inadequate expression of antibiotic resistance in a short time.

2. Monoclonal colony selection.
   a. Pick monoclonal colony from incubated agar plate and inoculated them in Terrific Broth (TB) medium (100 mL) with ampicillin (50 μg/mL).
   b. Shake the monoclonal colony medium at 37°C at 200 rpm for 12 h.
3. Monoclonal expansion and induction.
   a. Transfer 20 mL overnight culture into 1 L TB medium containing ampicillin (50 μg/mL) to expand culture.
   b. Cultivate it in the flask at 37 °C at 200 rpm until OD600 reaches 0.6–0.8.
   c. Induce the culture with 0.1 mM sterile IPTG at 16°C[4] at 220 rpm for 18–20 h.
4. Harvest of the induced cells.
   a. Collect the cell pellets following centrifugation at 8,980 × *g* for 10 min at 4°C (Beckman, Avanti JXN-26).
   b. Resuspend and oscillate the cell pellets thoroughly in 30 mL resuspension buffer (40 mM Tris (pH 7.5), 500 mM NaCl, 20 mM Imidazole, and 5% Glycerol).

c. Store at −80°C (for no longer than 1 month) or −20°C (for no longer than 1 week) until protein purification.

Note: Fresh cell pellets are recommended for immediate purification procedure due to likely higher protein yield and maintenance of protein activity.

LtCas12a protein purification

Timing: 3 days

Purification of LtCas12a could be carried out through affinity chromatography, ion exchange chromatography, and gel filtration chromatography. To obtain active LtCas12a, the following steps should be paid attention to.

5. Cell lysis.
   a. Add 0.35 mL PMSF (100 mM) and 4.90 μL β-Mercaptoethanol (14.3 M) into the cell suspension (total suspension volume is approximately 35 mL, including cell pellets and 30 mL resuspension buffer) to reach the final concentration of 1 mM PMSF and 2 mM β-Mercaptoethanol.
   b. Transfer the cell suspension to a high-pressure homogenizer (JNBIO, JN-2.5, Beijing, China) for cell disruption under the pressure of 1000 bar at 4°C.
   c. Perform cell crushing (2 s ultrasonication and 4 s pause in each cycle for 15 min with a power setting of 300 W) on ice using a probe sonicator (Scientz, JY92-IIN, Ningbo, China).
   d. Centrifuge the sample at 39,190 × *g* at 4°C for 1.5 h and collect the lysate supernatant.
   e. Filter the supernatant through 0.45 μm filter paper (PALL Corporation, USA) or 0.45 μm MILLEX-HV syringe filter (Merck Millipore).

Note: For maintenance of LtCas12a nuclease activity, all the protein purification procedures are performed on the ice or at 4°C after the cell lysis.

⚠ CRITICAL: PMSF (protease inhibitor) must be added to lysis buffer before cell lysis to minimize damage to the target protein. Moreover, the reducing agent β-Mercaptoethanol is needed to prevent proteins from forming intermolecular or intramolecular disulfide bonds.

6. Nickel affinity chromatography.
   a. Equilibrate gravity flow column packed with 2 mL Ni^{2+}-NTA-agarose (Cytiva, Ni Sepharose 6 Fast Flow) with 10 column volumes equilibration buffer (40 mM Tris (pH 7.5), 500 mM NaCl, 20 mM Imidazole, 5% Glycerol, and 2 mM β-Mercaptoethanol) at 4°C.
   b. Load the supernatant onto a pre-equilibrated gravity flow column and collect the flow-through fraction.
   c. Wash the column with 15–20 column volumes of washing buffer 1 (40mM Tris (PH 7.5), 500 mM NaCl, 20 mM Imidazole, 5% Glycerol, and 2 mM β-Mercaptoethanol) at 4°C.
   d. For target protein generation, conduct the elution step directly with 15 mL elution buffer 1 (40 mM Tris (pH 7.5), 500 mM NaCl, 300 mM Imidazole, 5% Glycerol, and 2 mM β-meter) at 4°C.

⚠ CRITICAL: To comprehensively evaluate the expression and purification of LtCas12a, all the products generated by each step must be retained for SDS-PAGE. In addition, this step may avoid complete protein loss due to operational mistakes.

7. Dialysis and digestion.
   a. For dialysis, place the eluted product into a dialysis bag (Asegene, Guangdong, China) having an appropriate molecular-weight cut-off membrane (such as 20kDa).

b. Prior to dialysis, add 1 mg TEV protease (produced by our lab) into the mixture contained in the dialysis bag[5] to remove the 6xHis-MBP tag.
c. Seal the dialysis apparatus at both ends and incubate the assembly in a 2 L beaker filled with dialysis buffer (20 mM Tris (pH 7.5), 250 mM NaCl, 5% Glycerol, and 2 mM β-Mercaptoethanol) with a stir bar to complete digestion and dialysis for 24 h at 4°C.
d. Analyze all the products generated by each step through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

8. A second run of Nickel affinity chromatography.[6]
   a. Load the dialysis solution onto the Ni^{2+}-NTA resin column and collect the flow-through fraction (targeted protein without His tag).
   b. Wash the column with washing buffer 2 (20 mM Tris (pH 7.5), 250 mM NaCl, 20 mM Imidazole, 5% Glycerol, and 2 mM β-Mercaptoethanol).
   c. Elute the column with elution buffer 2 (20 mM Tris (pH 7.5), 250 mM NaCl, 500 mM Imidazole, 5% Glycerol, and 2 mM β-Mercaptoethanol).
   d. Conduct the SDS-PAGE analysis for all the step products.

Note: When loading solution onto Ni^{2+}-NTA resin column, gentle dripping is recommended to avoid protein combination dysfunction caused by the uneven force of the resin.

9. Ion exchange chromatography (IEC).
   a. Equilibrate SP Sepharose HP cation exchange column (Cytiva, Marlborough, MA, USA) with 5–10 column volumes buffer A (20 mM Tris (pH 7.5), 250 mM NaCl, and 5% Glycerol).
   b. Pump the protein solution into the column for purification with the flow rate of 2.5 mL/min using a pure protein purification system (Inscinstech, Unique AutoPure100).
   c. Perform additional purification by column elution with a continuous linear NaCl gradient from 0.25 to 1 M concentrations (buffer A: 20 mM Tris (pH 7.5), 250 mM NaCl, and 5% Glycerol; buffer B: 20 mM Tris (pH 7.5), 1 M NaCl, and 5% Glycerol) at 2.5 mL/min flow rate for 50 min.
   d. Collect the eluents using an automatic fraction collector, 1.8 mL per EP tube named from A1 to A15, B1 to B15, C1 to C15, etc.
   e. Analyze the peak fractions by SDS-PAGE.
10. Gel filtration chromatography.
   a. Pooled the peak fractions from the ion exchange chromatography together.
   b. Concentrate protein product to 2 mL via 100 kDa ultrafiltration tube and centrifugation (3000g, 4°C).
   c. Equilibrate Superdex200 pg 16/200 gel filtration column (Cytiva, Marlborough, MA, USA) with 1.5 column volumes size exclusion chromatography (SEC) buffer (20 mM Tris (pH 7.5), 250 mM NaCl, and 2 mM β-Mercaptoethanol).
   d. Use the protein purifier (Inscinstech, Unique AutoPure100) for further protein purification and inject the concentrated protein product into the gel filtration column.
   e. Wash the column with SEC buffer (20 mM Tris (pH 7.5), 250 mM NaCl, and 2mM β-Mercaptoethanol).
   f. Collect the eluents using an automatic fraction collector, 1.8 mL per EP tube named from A1 to A15, B1 to B15, C1 to C15, etc.
   g. Analyze the peak fractions by SDS-PAGE.
11. Protein concentrating and concentration measurement.
   a. Concentrate protein product to targeted protein concentration via 100 kDa ultrafiltration tube and centrifugation (3000g, 4°C).
   b. Measure the final LtCas12a protein concentration using bicinchoninic acid (BCA) protein assay and store it at −80°C.
   c. Add the storage buffer (20 mM Tris (pH 7.5), 250 mM NaCl, and 5% Glycerol) into the concentrated protein solution to get the required concentration.

LtCas12a protein validation

Timing: 1 day

After purification and concentration measurement of LtCas12a, functional validation is further performed via three distinct methods. Since LtCas12a is capable of the target DNA cleavage and indiscriminately collateral cleavage of ssDNA, the assessment of LtCas12a could be achieved by cleavage assay *in vitro*,[7] DETECTR fluorescence detection,[8] and lateral flow assay (LFA).[9] The details are described below.

12. Functional validation of LtCas12a by cleavage assay *in vitro*.
    a. Incubate the *in vitro* cleavage mixture containing 0.3 μL LtCas12a (10 μM), 0.3 μL crRNA (10 μM), and 1 μL 10× NEBuffer 2.1 (final concentrations of 0.3 μM LtCas12a and 0.3 μM crRNA) at 37°C for 10 min.
    b. Add 200 ng dsDNA substrates into the reaction system (the total system volume was 10 μL) with incubation at 37°C for 15 min.
    c. Digest the reaction mixture with proteinase K for 15 min, and then subject it to agarose gel electrophoresis (AGE).
13. Functional validation of LtCas12a by DETECTR assay.
    a. For ribonucleoproteins (RNPs) formation, incubate 2 μL LtCas12a (10 μM), 2 μL crRNA (10 μM), and 2 μL 10× NEBuffer 2.1 at 37°C for 10 min.[10,11]
    b. Add 5′-FAM-ssDNA-BHQ-3′ reporter (Sangon, China) (the final concentration is 25–350 nM) and target dsDNA into the RNP mixture (the total system volume is 20 μL).
    c. Read and record the fluorescence signal (5′-FAM-ssDNA-BHQ-3′ reporter, λex: 485 nm; λem: 535 nm) at 37°C using a qPCR machine (BIOER, Hangzhou, China).

⚠ CRITICAL: Insufficient incubation time of LtCas12a and crRNA may affect the LtCas12a cleavage efficiency, leading to the failure of the experiment.

14. Functional validation of LtCas12a by lateral flow strip assay.
    a. Incubate 2 μL LtCas12a (10 μM), 2 μL crRNA (10 μM), and 2 μL 10× NEBuffer 2.1 at 37°C for 10 min to form RNP.[10,11]
    b. Add the 5′-FAM-ssDNA (TTATT)-Biotin-3′ reporter (Sangon, China) (the final concentration is 350 nM) and target dsDNA into the RNP mixture (the total volume is 20 μL).
    c. Incubated the above solution at 37°C for 30–50 min.
    d. Mix the 20 μL reaction with 80 μL strip buffer (Milenia Biotec GmbH, German) for 10 min.
    e. Load onto the strip (Milenia Biotec GmbH, German) and keep it at 25°C for 5 min.
    f. Photograph and record the result and read the grayscale values of bands using ImageJ software.[12]

Note: Once LtCas12a recognizes and cleavages the target dsDNA, the 5′-FAM-ssDNA-Biotin-3′ reporter will be indiscriminately cleaved, resulting in ssDNA-Biotin-3′ captured on the control line while the rest of the reporter 5′-FAM-ssDNA with golden nanoparticles (GNPs) was captured by anti-GNPs antibody to generate a visible test line.

EXPECTED OUTCOMES

This protocol describes a feasible and stable method for cloning, expression, purification, and functional validation of LtCas12a. The LtCas12a is purified through Nickel affinity chromatography, a second run of Nickel affinity chromatography, ion exchange chromatography, and gel filtration chromatography (Figure 1). The dsDNA cleavage activity and ssDNA collateral cleavage are validated in cleavage assay *in vitro*, DETECTR fluorescence detection, and lateral flow strip assay (Figure 2).

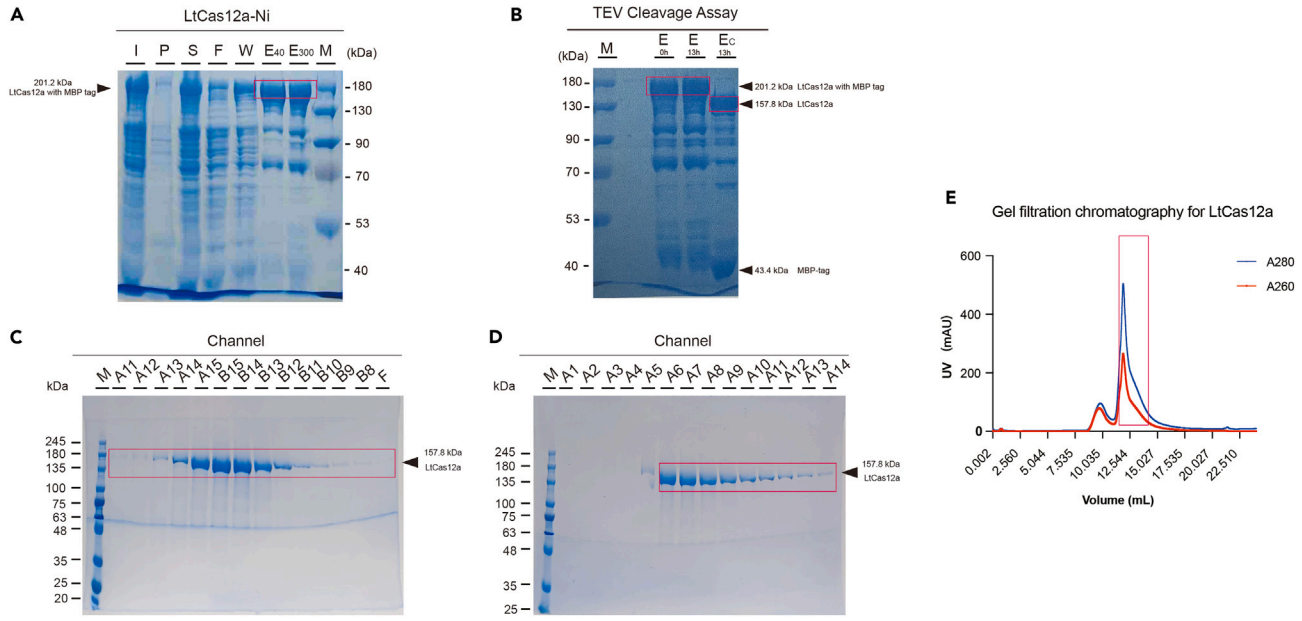

Figure 1. Purification profiles for LtCas12a

(A) Purification of LtCas12a using Nickel affinity chromatography. I, Bacteria suspension after cell crushing; P, Pellet; S, Supernatant; F, Flow-through; W, Wash; E_{40}, Elution with buffer containing 40 mM Imidazole; E_{300}, Elution with buffer containing 300 mM Imidazole; M, Protein marker.

(B) TEV proteinase cleavage assay for a second run of Nickel affinity chromatography. E, Elution; Ec, Elution (after cleavage with TEV proteinase); M, Protein marker.

(C) Purification of LtCas12a using ion exchange chromatography. With a continuous linear NaCl gradient elution, fractions were collected using an automatic fraction collector (1.8 mL per tube). The peak fractions were collected from A11–A15 and B8–B15 tubes for SDS-PAGE. F, Flow-through after pumping the protein product into the ion exchange column. SDS-PAGE analysis was performed at constant voltage (280V) for 30 min.

(D) Purification of LtCas12a using gel filtration chromatography. Fractions were collected using an automatic fraction collector (1.8 mL per tube). The fractions were collected from A1–A14 tubes for SDS-PAGE (280 V, 30 min).

(E) Gel filtration chromatogram for LtCas12a. The red line stands for the absorbance at 260 nm. The blue line stands for the absorbance at 280 nm. Fractions highlighted in red correspond to the fractions highlighted in red in Figure 1D.

LIMITATIONS

This protocol provides a detailed strategy for LtCas12a generation and purification. With 6xHis-MBP tag, LtCas12a could be purified by Nickel affinity chromatography. However, removing 6xHis-MBP tag by TEV protease is time-consuming, for about 36 h. The utility of other tags such as His-SUMO (only takes 2 h to remove the tag)[13,14] may greatly improve LtCas12a production.

TROUBLESHOOTING

Problem 1

No or few colonies for agar plate containing ampicillin (step 2).

Potential solution

Confirm the plasmids by Sanger sequencing and repeat the transformation with fresh reagents. Sometimes, excessive ampicillin concentration in agar plates could result in the failure of colony formation.

Problem 2

Failure of monoclonal expansion (step 3).

Potential solution

Repeat monoclonal selection and confirm successful transformation into the competent *E. coli* BL21 (DE3) cells by Sanger sequencing.

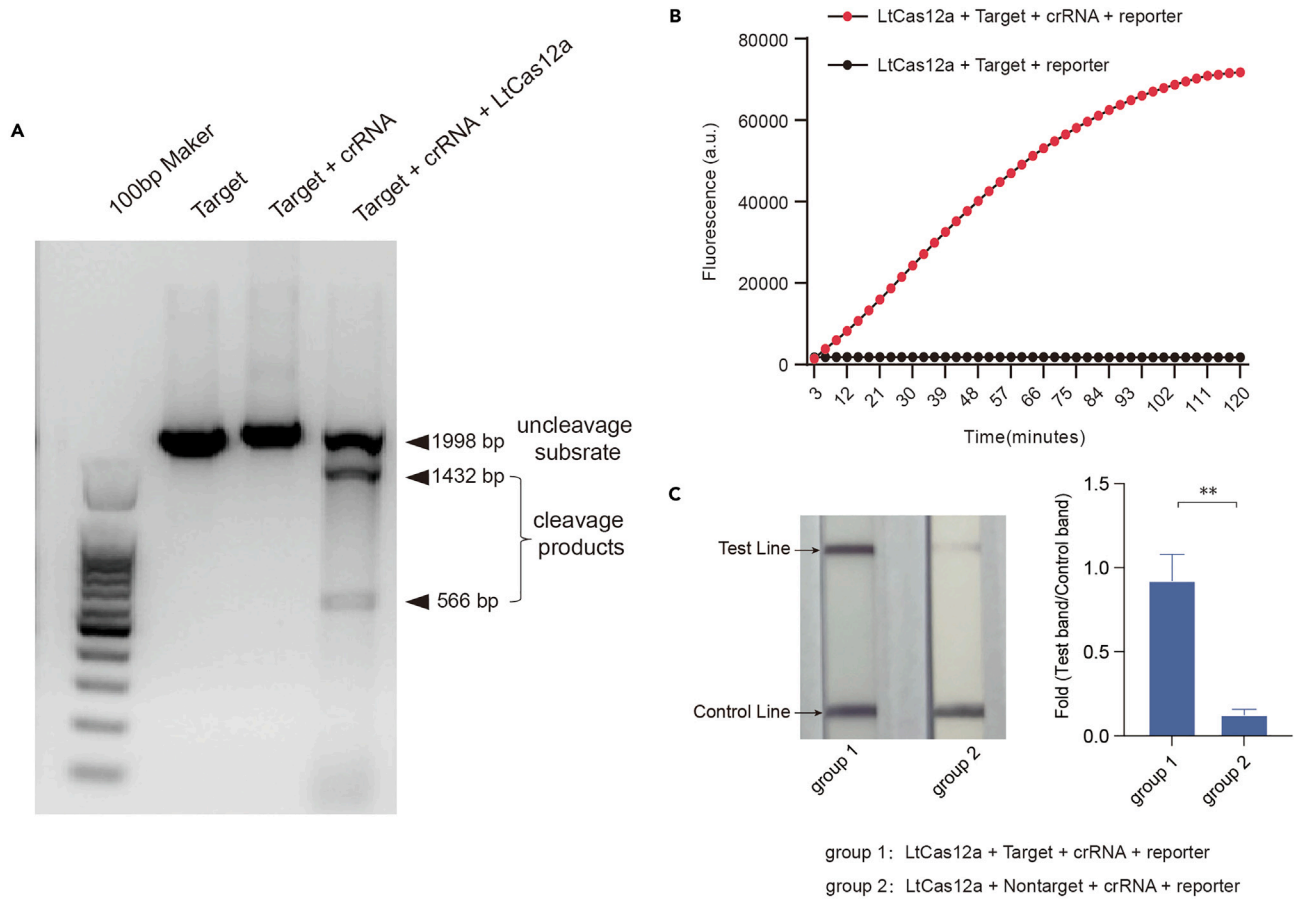

Figure 2. Functional validation for LtCas12a

(A) Functional validation of LtCas12a by cleavage assay *in vitro*. 1998 bp dsDNA was used as the target. After 15 min incubation of the mixture, the product was stained with 6× DNA loading buffer and then used for agarose gel electrophoresis at 120 V for 30min. Cleavage products (1432bp band and 566 bp band) were observed in the presence of target+crRNA+LtCas12a, indicating that LtCa12a is active for cis-cleavage activity (dsDNA cleavage).

(B) Functional validation of LtCas12a by DETECTR fluorescence assay. The x-axis represented time, and the y-axis represented fluorescence signal intensity. QPCR machine was used for recording fluorescence signal (5′-FAM-ssDNA-BHQ-3′ reporter, λex: 485 nm; λem: 586 nm). After incubation of the mixture (LtCas12a, Target, crRNA, and reporter), the increased fluorescence intensity (red-dotted line) was observed, indicating trans-cleavage activity of LtCas12a (indiscriminate ssDNA cleavage after dsDNA).

(C) Functional validation of LtCas12a by lateral flow strip assay. HPV 16 L1 gene was used as the target. 5′-FAM-ssDNA-Biotin-3′ was utilized as the reporter. The test result was positive when both the control line and test line were present (indicating the occurrence of indiscriminate ssDNA cleavage) and negative when only the control line was observed. The corresponding adjusted band intensity was analyzed by ImageJ. Data from biological replicates were shown as mean ± s.e.m. (N = 3). One-way ANOVA and Bonferroni test, *P < 0.05, **P < 0.01, ***P < 0.001, ns, not significant.

Problem 3

Low expression of target protein in the supernatant after cell harvest and cell lysis (step 5).

Potential solution

Numerous reasons could result in the imperfect production of protein in the supernatant. The attempts below could be in consideration: a. improvement of induction conditions (change the induction temperature and time); b. application of protease inhibitors (application of PMSF); c. obtaining more soluble protein by reducing the formation of inclusion bodies which would lead to protein precipitation in cell pellets (attempt lower induction temperature, reduce IPTG concentration, and extend the induction time).

Problem 4

Low elution efficiency during Nickel affinity chromatography (step 6).

Potential solution

To improve elution efficiency, perform a second round of elution or attempt elution buffer with increasing concentrations of imidazole.

Problem 5

Inefficient LtCas12a cleavage and DETECTR fluorescence signal (step 12 or step 13).

Potential solution

Optimize the purity and concentration of LtCas12a protein and increase the LtCas12a amount in the reaction mixture. Of note, improper preservation of LtCas12a (using ddH_2O as the storage buffer or storing the protein at room temperature) may cause inactivity of LtCas12a.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Hu Zheng (huzheng1998@163.com).

Materials availability

This study did not generate any unique reagents. The commercially available reagents used in this study are provided in the key resources table.

Data and code availability

This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102600.

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AUTHOR CONTRIBUTIONS

Investigation, B.Z., Y.C., and Y.W.; methodology, B.Z., Y.C., Z.Hu, and Z.L.; validation, B.Z. and Y.C.; writing-original draft, B.Z., Y.C., L.L., Z.Hu, and Z.L.; writing-review & editing, Y.C.; visualization, L.L., J.L., and Z.Huang; project administration, Z.Hu, R.T., and Z.L.; supervision, Z.Hu, R.T., and Z.L.; conceptualization, Z.Hu; funding acquisition, Z.Hu. All authors have read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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