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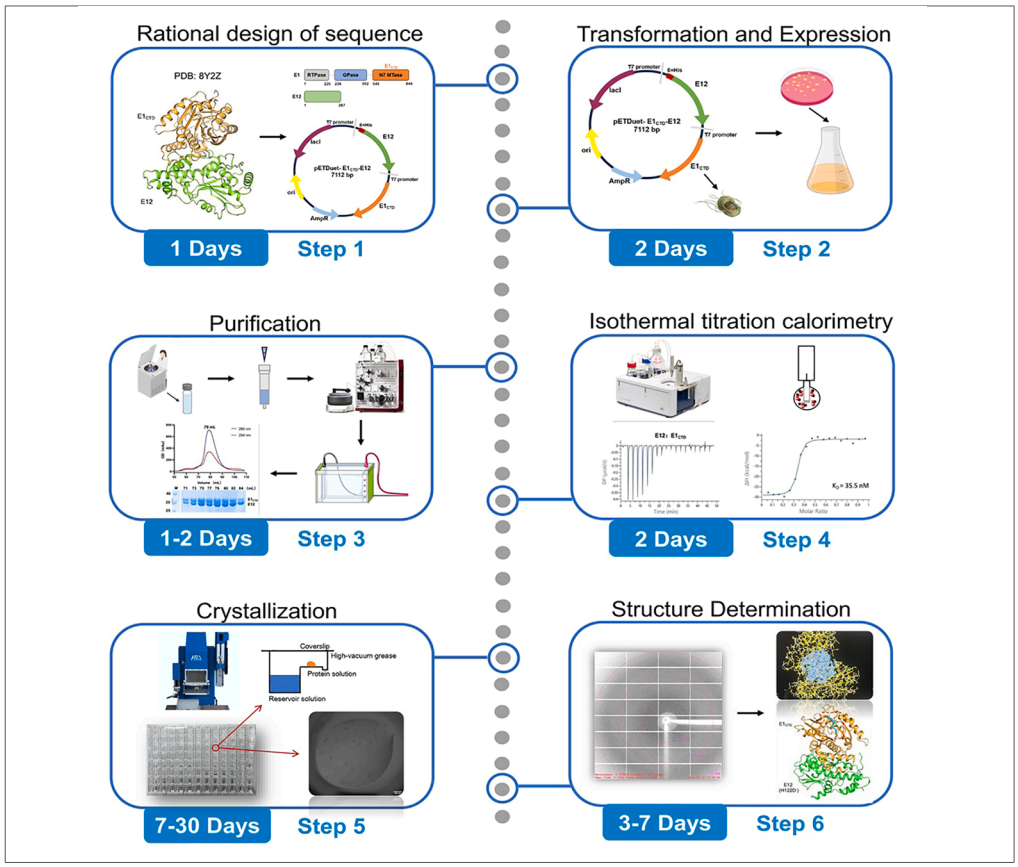
Protocol for purification, crystallization, and structure determination of the mpox virus mRNA cap N7 methyltransferase complex



The mpox virus (MPXV) mRNA cap N7 methyltransferase (RNMT) methylates guanosine at mRNA 5′-cap N7 positions to enable immune evasion. Here, we present a protocol for $E1_{CTD}$-E12 complex purification and crystallization. We describe steps for rational sequence design of the complex, co-expression in *E. coli*, affinity chromatography purification, gel filtration, and crystallization optimization using vapor diffusion. We further outline X-ray diffraction data collection and structure determination. This reproducible framework enables structural analysis of viral mRNA-modifying enzyme complexes.

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

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Highlights

Rational design of $E1_{CTD}$-E12 co-expression system

Purification complexes by affinity chromatography and gel filtration chromatography

Optimization of SAM-bound $E1_{CTD}$-E12 complex crystallization via mutant construction

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Protocol for purification, crystallization, and structure determination of the mpox virus mRNA cap N7 methyltransferase complex

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SUMMARY

The mpox virus (MPXV) mRNA cap N7 methyltransferase (RNMT) methylates guanosine at mRNA 5′-cap N7 positions to enable immune evasion. Here, we present a protocol for $E1_{CTD}$-E12 complex purification and crystallization. We describe steps for rational sequence design of the complex, co-expression in *E. coli*, affinity chromatography purification, gel filtration, and crystallization optimization using vapor diffusion. We further outline X-ray diffraction data collection and structure determination. This reproducible framework enables structural analysis of viral mRNA-modifying enzyme complexes.

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

BEFORE YOU BEGIN

The global outbreak of mpox, caused by the mpox virus (MPXV), has drawn international attention, emerging as a significant infectious disease event following COVID-19. Throughout poxvirus-host coevolution, viral mRNA has evolved to mimic host mRNA by adopting the same cap structure, enabling immune evasion and efficient viral protein synthesis.[2] MPXV, a member of the poxvirus family, encodes a heterodimeric RNA capping enzyme composed of a large subunit, E1, and a small subunit, E12.[3] However, the precise mechanism by which E1 and E12 assemble remains poorly understood. Understanding the structural assembly of the MPXV E1-E12 complex is crucial for elucidating viral RNA "camouflage" strategies that facilitate immune escape. However, the purification of multi-subunit complexes presents significant challenges in maintaining structural integrity. To address this, we develop a co-expression system for the $E1_{CTD}$ and E12 subunits, allowing us to successfully resolve the three-dimensional structure of the E12/$E1_{CTD}$-E12-SAM ternary complex. This methodological framework not only advances our understanding of MPXV RNA capping but also provides a broadly applicable approach for crystallographic studies of other oligomeric protein assemblies.

This protocol outlines a detailed workflow for the purification and crystallization of the mpox virus (MPXV) mRNA capping enzyme subunits, with a particular focus on the E12 subunit and its complex with $E1_{CTD}$. Designed as a practical guide for crystallographers, the procedure is divided into three key stages. In the first stage, we describe the expression and purification of $E1_{CTD}$, E12, and the $E1_{CTD}$-E12 complex from Escherichia coli cells. Efficient protein yields are achieved by employing a high-salt buffer during cell lysis and affinity purification steps, followed by size-exclusion

chromatography (SEC) for further purification. The second stage covers the crystallization screening of purified E12 and $E1_{CTD}$-E12, both in their apo forms and in complex with S-adenosylmethionine (SAM) and guanosine triphosphate (GTP). Finally, in the third stage, we use isothermal titration calorimetry (ITC) to characterize intermolecular interactions within the capping apparatus. Our findings highlight the essential role of the E12 subunit in substrate recognition and SAM binding, which is crucial for the enzymatic activity of the capping complex. This protocol provides a reproducible framework for structural studies of viral RNA modification enzymes and other oligomeric protein assemblies.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli DH5α chemically competent	Sangon Biotech	B528413-0100
E. coli BL21(DE3)	Sangon Biotech	B528414-0100
Chemicals, peptides, and recombinant proteins		
Kanamycin	Sangon Biotech	A506636-0100
Chloramphenicol	BBI	CB0118
Ampicillin	BBI	A610029-0025
Isopropyl-beta-D-thiogalactoside (IPTG)	BBI	A600168-0100
Imidazole	BBI	A600277-05
XhoI	Takara	Cat#1094A
BamHI	Thermo Scientific	Cat#FD0054
DpnI	Thermo Scientific	Cat#FD1703
LB broth, Miller	Becton, Dickinson and Company	Ref.214906
LB agar, Miller	Sangon Biotech	A507003-0250
Tris-HCl	Solarbio	T8060
Sodium chloride	BBI	A610476-0005
Ulp1 protease	This paper	N/A
Ni-NTA beads	Smart-Lifesciences	Cat#SA005005
GTP	BBI	A620332-0250
S-adenosine-L-methionine(SAM)	Aladdin	S192607-5mg
Glycerol	Sinopharm Chemical Reagent	10010618
Critical commercial assays		
PrimeSTAR Max DNA polymerase	Takara	R045A
ClonExpress Ultra one step cloning kit	Vazyme	C117-01
HiPure gel pure DNA mini kit	Magen	D2111-03
Index kit	Hampton Research	HR2-134
Crystal screen kit	Hampton Research	HR2-138
Wizard Classic 1/2	Rigaku	1009530,1009531
Wizard Classic 3/4	Rigaku	1009532,1009533
Oligonucleotides		
Primer:$E1_{CTD}$ WT primer-forward : agagaacagattggtggatccatggataaa tttcgtctgaatccg	Tsingke	N/A
Primer:$E1_{CTD}$ WT primer-reverse : cagtggtggtggtggtggtgctcgagttaac gtttgctaaaaacataaacaac	Tsingke	N/A
Primer: pETDuet-$E1_{CTD}$ -E12 primer-forward1 : ccatcatcaccacagccaggatccgatggatgaaatcgt taaaaacatccgc	Tsingke	N/A
Primer: pETDuet-$E1_{CTD}$ -E12 primer-forward2 : taagtcgacaagcttgcg	Tsingke	N/A
Primer: pETDuet-$E1_{CTD}$ -E12 primer-forward3 : gtataagaaggagatatacatatggataaatttcgtctgaatccg	Tsingke	N/A
Primer: pETDuet-$E1_{CTD}$ -E12 primer-reverse1 : gccgcaagcttgtcgacttacagcagcagtttcaccaggc	Tsingke	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer: pETDuet-E1$_{CTD}$ -E12 primer-reverse2 : atgtatatctccttcttatacttaactaatatac	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$ -E12 primer-reverse3 : gcagcggtttctttaccagactcgagttaacgtttgctaaaaacataaacaac	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$ -E12(H122D) primer-forward : tacaggttattttcggtca gatcattgttgcttttaattgcatcac	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$ -E12(H122D) primer-reverse : gtgatgcaattaaaagcaa caatgatctgaccgaaaataacctgta	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$(D587N)-E12 primer-forward : atgtattgtagcaaaacc tttctgaatgatagcaataaacgtaaagttc	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$(D587N)-E12 primer-reverse : gaactttacgtttattgcta tcattcagaaaggttttgctacaatacat	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$ - E12(R49A) primer-forward : caaattattttctgcagat cagcgctgttaatgatctgaatcgtatgc	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$ -E12(R49A) primer-reverse : gcatacgattcagatcatt aacagcgctgatctgcagaaaataatttg	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$(L586A)-E12 primer-forward : gtttattgctatcatccgc aaaggttttgctacaatacatgctaatcagca	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$(L586A)-E12 primer-reverse : tgctgattagcatgtattg tagcaaaacctttgcggatgatagcaataaac	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$ -E12(F44A) primer-forward : tattccaggctcggcgc cgggcttttacccag	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$ -E12(F44A) primer-reverse : ctgggtaaaagcccgg cgccgagcctggaata	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$(L575A)-E12 primer-forward : tttgctacaatacatgcta atcagcgcggttttaacataattgctcagaata	Tsingke	N/A
Primer: pETDuet-E1$_{CTD}$(L575A)-E12 primer-reverse : tattctgagcaattatgtta aaaccgcgctgattagcatgtattgtagcaaa	Tsingke	N/A
Recombinant DNA		
pSMT3-WT E1$_{CTD}$	This study	N/A
pSMT3-WT E12	This study	N/A
pETDuet-E12-E1$_{CTD}$	This study	N/A
pETDuet-E1$_{CTD}$ -E12(H122D)	This study	N/A
pETDuet-E1$_{CTD}$(D587N)-E12	This study	N/A
pETDuet-E1$_{CTD}$ -E12(R49A)	This study	N/A
pETDuet-E1$_{CTD}$(L586A)-E12	This study	N/A
pETDuet-E1$_{CTD}$ -E12(F44A)	This study	N/A
pETDuet-E1$_{CTD}$(L575A)-E12	This study	N/A
pETDuet-E1$_{CTD}$(L586A)-E12(F44A)	This study	N/A
Software and algorithms		
Prism 9.0	GraphPad	http://www.graphpad.com
PyMol 2.3	Schrodinger	pymol.org
Phenix	Adams et al., 2010	http://www.phenix-online.org/
COOT	Emsley et al., 2010	http://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot
Other		
Amicon Ultra-15 centrifugal filter unit (30 kDa MWCO)	Millipore	Cat#UFC903096
NanoDrop 2000	Thermo Scientific	ND-2000
D-Tube dialyzers (15 mL, MWCO 3.5 kDa)	Millipore	Cat#71742

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Magnetic stirrer	Corning	PC-410
ÄKTA pure 25 M protein purification system	Cytiva	Cat#29018226
Superdex 200 10/300 GL	Cytiva	Cat#17517501
HiLoad 16/600 Superdex 200 prep grade	Cytiva	Cat#28989335
ITC injection syringe (syringe filling, ITC200)	Malvern Panalytical	SYN50018P

MATERIALS AND EQUIPMENT

Lysis buffer		
Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (2 M)	50 mM	25 mL
NaCl (5 M)	500 mM	100 mL
Imidazole (5 M)	10 mM	2 mL
ddH_2O	N/A	873 mL
Total	**N/A**	**1,000 mL**
Store at 4°C within 6 months.		

Wash buffer		
Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (2 M)	50 mM	2.5 mL
NaCl (5 M)	500 mM	10 mL
Imidazole (5 M)	50 mM	1 ml
ddH_2O	N/A	86.5 mL
Total	**N/A**	**100 mL**
Store at 4°C within 6 months.		

Elution buffer		
Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (2 M)	50 mM	2.5 mL
NaCl (5 M)	500 mM	10 mL
Imidazole (5 M)	500 mM	10 ml
ddH_2O	N/A	77.5 mL
Total	**N/A**	**100 mL**
Store at 4°C within 6 months.		

Dialysis buffer		
Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (2 M)	50 mM	50 mL
NaCl (5 M)	500 mM	200 mL
ddH_2O	N/A	1750 mL
Total	**N/A**	**2000 mL**
Store at 4°C within 6 months.		

Size exclusion chromatography (SEC) buffer		
Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (2 M)	50 mM	12.5 mL
NaCl (5 M)	100 mM	10 mL
ddH_2O	N/A	477.5 mL
Total	**N/A**	**500 mL**
Store at 4°C within 6 months.		

Isothermal titration calorimetry (ITC) buffer		
Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (2 M)	50 mM	2.5 mL
NaCl (5 M)	100 mM	2 mL
ddH_2O	N/A	95.5 mL
Total	**N/A**	**100 mL**

Store at 4°C within 6 months. Filter right before use and equilibrate the temperature before start experiment.

STEP-BY-STEP METHOD DETAILS

Generation of $E1_{CTD}$, $E1_{CTD}$-E12, and $E1_{CTD}$-E12 mutant plasmid constructs

Timing: 2 days

This section describes the construction of wild-type and mutant plasmid vectors for $E1_{CTD}$, co-expression of $E1_{CTD}$ and E12 subunits, including PCR, homologous recombination, and bacterial transformation. (Figure 1 shows the construction methods of $E1_{CTD}$, $E1_{CTD}$-E12 and mutant plasmids.)

1. PCR amplification of target sequences.
   a. Prepare PCR reactions with the following components.

PCR reaction master mix	
Reagent	Amount
DNA template	1 μL (50 ng)
Forward primer	1 μL (10 pmol)
Reverse primer	1 μL (10 pmol)
PrimeSTAR Max Premix (2X)	25 μL
ddH_2O	22 μL

Total reaction volume in each tube should be 50 μL. See Table 1.

   b. Run PCR programs.

PCR cycling conditions for pSMT3-WT $E1_{CTD}$ and pETDuet-E12-$E1_{CTD}$ troubleshooting 1			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	30 s	38 cycles
Annealing	57°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	1
Hold	4°C	indefinitely	

See Table 2.

PCR cycling conditions for pETDuet-E12-$E1_{CTD}$ mutants			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	30 s	18 cycles
Annealing	57°C	30 s	
Extension	72°C	3 min	
Final extension	72°C	5 min	1
Hold	4°C	forever	

See Table 3.

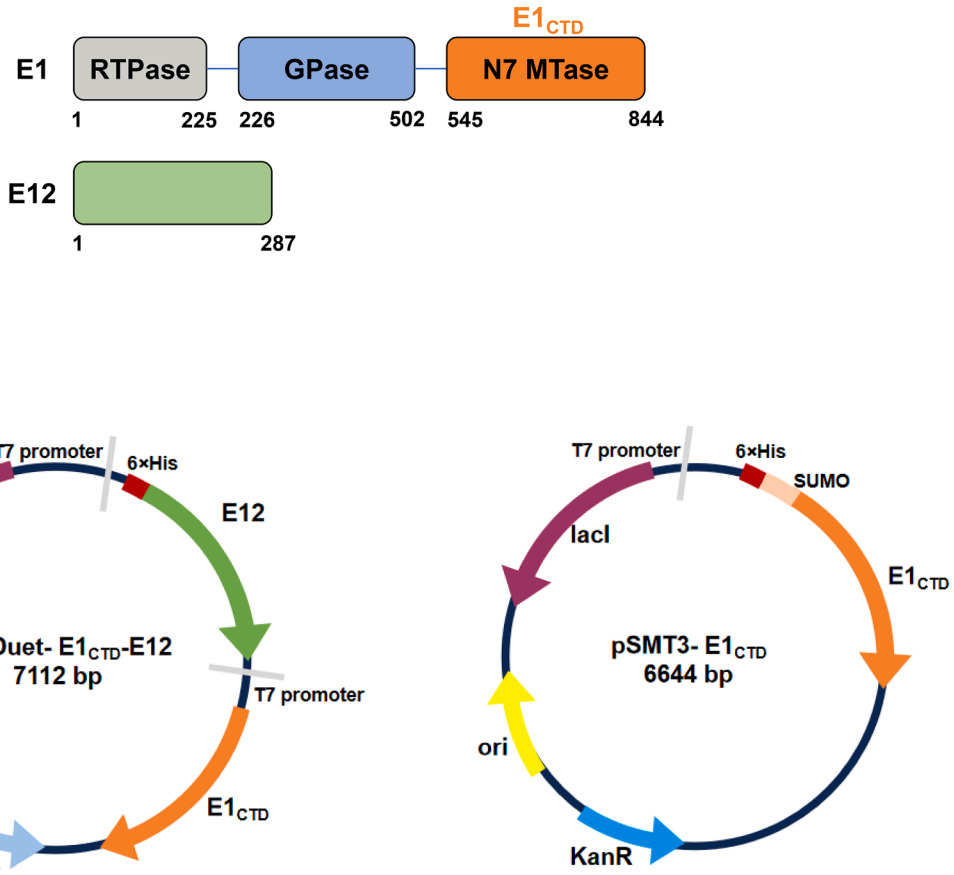

Figure 1. $E1_{CTD}$, $E1_{CTD}$-E12 and $E1_{CTD}$-E12 mutants plasmid constructs

The N7 MTase domain (denoted as $E1_{CTD}$) of MPXV large subunit E1 and a small subunit E12 and are colored in orange and green respectively.

Note: Engineer the pSMT3 plasmid from the pET28a plasmid series and optimize it specifically for SUMO (Small Ubiquitin-like Modifier) fusion protein expression and purification. Design the pETDuet-1 plasmid (71146-3, Merck) to coexpress two target genes. Encode two multiple cloning sites in the vector, ensure each site is preceded by a T7 promoter, lac operator, and ribosome binding sites.

2. Gel purification and homologous recombination.
   a. Separate the PCR products by agarose gel electrophoresis at 100 V for 30 min.
   b. Excise the target DNA fragments from the agarose gel and purify them using a commercial gel extraction kit according to the manufacturer's protocol.
   c. Perform homologous recombination.

The homologous recombination process proceeds through the following sequential steps	
Reagent	Amount
PCR product	1 μL
Plasmid vector	1.5 μL
2 X CE Mix	2.5 μL
50°C, 15 min	

See Table 4.

3. Bacterial transformation and plasmid validation (pSMT3-WT $E1_{CTD}$).

Table 1. Total PCR reaction master mix in each tube should be 50 μL

Reagent	Amount
DNA template	1 μL (50 ng)
Forward primer	1 μL (10 pmol)
Reverse primer	1 μL (10 pmol)
PrimeSTAR Max Premix (2X)	25 μL
ddH_2O	22 μL

a. Transform DH5α competent cells with the PCR products and plate onto LB agar plates with 50 μg/mL kanamycin.
b. Incubate at 37°C for 12–18 h.
c. Pick a single colony and inoculate it into 5 mL of LB medium supplemented with 50 μg/mL kanamycin.
d. Incubate at 37°C for 12–18 h.
e. Perform plasmid miniprep using the plasmid extraction kit.

4. Bacterial transformation and plasmid validation (pETDuet-E12-$E1_{CTD}$ and pETDuet-E12-$E1_{CTD}$ mutants).
   a. Add 1 μL of DpnI (10 U/μL, Thermo Scientific) and incubate at 37°C for 2 h.

   Note: Add DpnI enzyme only to mutant constructs.

   b. Transform DH5α competent cells with the PCR products and plate onto LB agar plates with 100 μg/mL ampicillin.
   c. Incubate at 37°C for 12–18 h.
   d. Pick a single colony and inoculate it into 5 mL of LB medium supplemented with 100 μg/mL ampicillin.
   e. Incubate at 37°C for 12–18 h.
   f. Perform plasmid miniprep using the plasmid extraction kit.

Expression of $E1_{CTD}$, E12, $E1_{CTD}$-E12, and mutants

Timing: 3 days

This section outlines bacterial transformation, large-scale protein expression, and cell harvesting steps to obtain biomass for downstream purification. (Figure 2A shows the expression processes of $E1_{CTD}$, $E1_{CTD}$-E12 and mutants.)

5. Bacterial transformation and plate culture.
   a. Thaw E. *coli* BL21(DE3) expression-competent cells from −80°C storage on ice for 5 min.
   b. Add 1 μL of expression plasmid to 100 μL BL21(DE3) competent cell suspension.
   c. Maintain ice incubation for 30 min.
   d. Apply thermal shock at 42°C for 45 s followed by 3-min ice recovery.
   e. Add 500 μL antibiotic-free LB medium and incubate at 37°C with 200 rpm shaking for 1 h.

Table 2. PCR cycling conditions for pSMT3-WT $E1_{CTD}$ and pETDuet-E12-$E1_{CTD}$

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	30 s	38 cycles
Annealing	57°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	1
Hold	4°C	Forever	

Table 3. PCR cycling conditions for pETDuet-E12-E1$_{CTD}$ Mutants

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	30 s	18 cycles
Annealing	57°C	30 s	
Extension	72°C	3 min	
Final extension	72°C	5 min	1
Hold	4°C	Forever	

f. Pellet cells by centrifugation at 1,370 × *g* (Micro CL17 21 microcentrifuge, 24 × 1.5 mL fixed-angle rotor, Thermo Fisher Scientific) for 1 min.
g. Resuspend pellet in 100 μL antibiotic-free LB and spread onto selective agar plates.
   i. Kanamycin plates (50 μg/mL) for pSMT3-E1$_{CTD}$/pSMT3-E12 constructs.
   ii. Ampicillin plates (100 μg/mL) for pETDuet-E1$_{CTD}$-E12.
h. Incubate inverted at 37°C for 12–18 h.

6. Starter culture and large-scale expression.
   a. Select 3–5 distinct colonies to inoculate 100 mL selective LB broth.

   Note: Use Erlenmeyer flasks as culture vessels and maintain the working volume at ≤20% of the total flask capacity to ensure adequate oxygen transfer.

   b. Culture at 37°C with 220 rpm shaking until turbidity develops (8–10 h).
   c. Transfer 15–20 mL starter culture to 1 L selective LB medium.
   d. Grow at 37°C until OD_{600} reaches 0.8–1.0.
   e. Acclimate cells at 18°C for 30 min.
   f. Induce protein expression by adding IPTG to 0.2 mM final concentration. Continue 18–20 h incubation at 18°C.
7. Cell harvesting and storage.
   a. Harvest cells by centrifugation at centrifuge at 5,420 ×g (5,000 rpm, rotor radius 194 mm, F9-6 x 1000 LEX fixed-angle rotor, Thermo Fisher Scientific) for 10 min.
   b. Immediate processing: resuspend pellet in ice-cold buffer.

   Note: For delayed processing, aliquot samples into 1 mL tubes, centrifuge, record biomass weight, snap-freeze in liquid nitrogen, and store at −80°C.

Purification of E1$_{CTD}$ and E12

Timing: 2 days

This section details the sequential purification of His-SUMO-tagged E1$_{CTD}$ and E12 subunits using affinity and size-exclusion chromatography (SEC), including tag cleavage and buffer optimization. (Figures 2B and 2C depict the purification processes of E1$_{CTD}$ and E12.)

8. Cell lysis and Ni-NTA affinity chromatography.

Table 4. Preparation and process of homologous recombination system

Reagent	Amount
PCR product	1 μL
Plasmid vector	1.5 μL
2 X CE Mix	2.5 μL
50°C, 15 min	

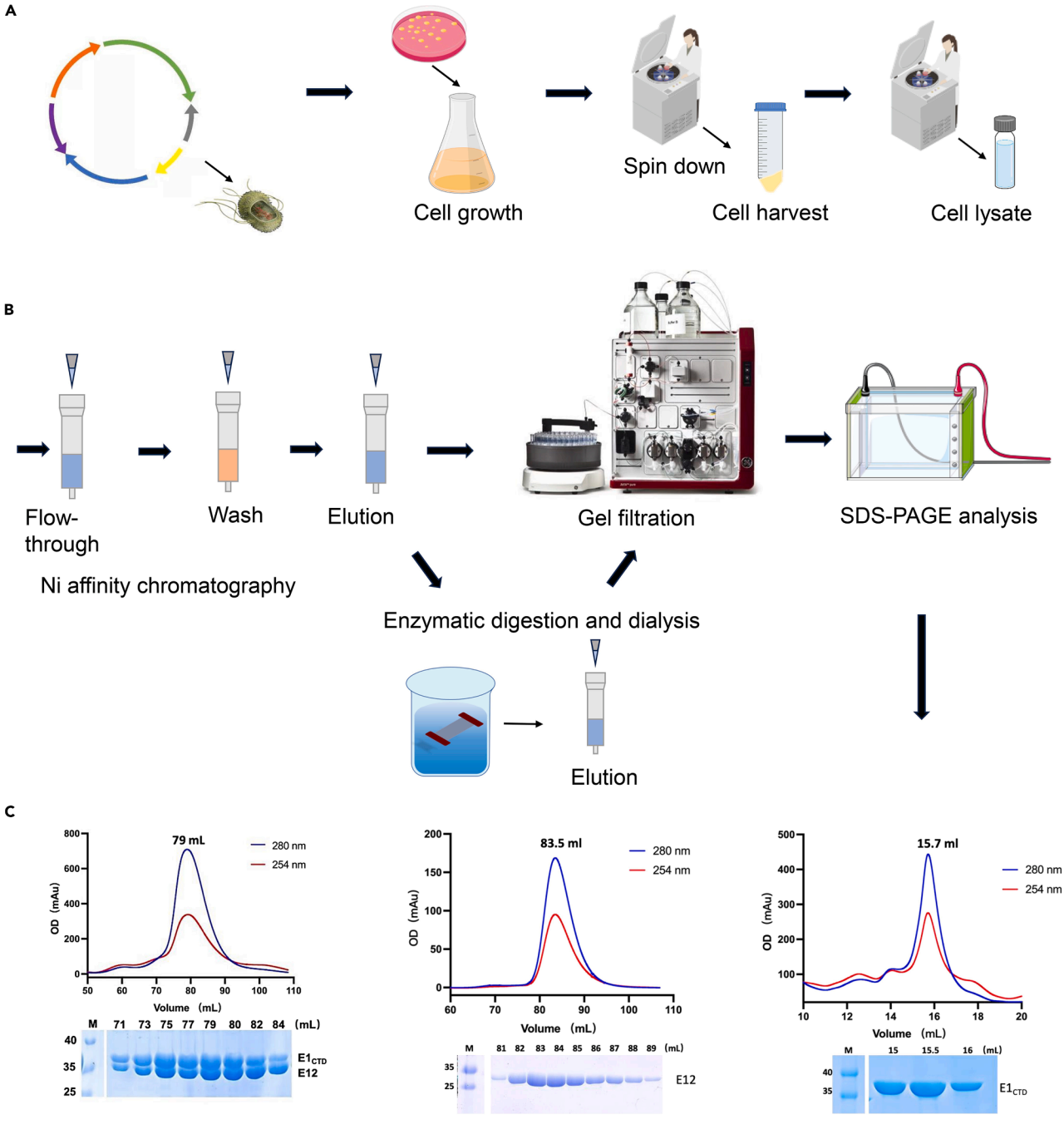

Figure 2. Diagram of the procedure for expression and purification of the MPXV $E1_{CTD}$, E12 and $E1_{CTD}$-E12 complex

(A) Preparation of cells expressing $E1_{CTD}$, E12 and $E1_{CTD}$-E12 complex.

(B) Affinity purification, removal of the His-SUMO tag and size exclusion chromatography of the $E1_{CTD}$, E12 and $E1_{CTD}$-E12 complex.

(C) The SDS-PAGE results of the $E1_{CTD}$, $E1_{CTD}$-E_{12} complex, E12, and different mutants, corresponding to size exclusion chromatography. M: protein marker.

a. Resuspend cell pellets (1 g wet weight) in 10 mL ice-cold lysis buffer using gentle pipetting to minimize bubble formation.

Note: Ensure complete homogenization without residual cell clumps.

b. Pre-cool the high-pressure homogenizer cooling system to 4°C. Activate the hydraulic pump and main unit, maintaining hydraulic pressure at 20 kg/cm^2 and operational pressure at 1000-1500 bar.
c. Perform sequential flushing protocol.
   i. 20% ethanol (2 cycles), deionized H_2O (2 cycles), lysis buffer equilibration (1 cycle).
   ii. Perform 3 sequential homogenization cycles.
   iii. Post-processing maintenance: H_2O flush (2 cycles), 20% ethanol preservation (2 cycles), seal inlet with 20% ethanol before system depressurization and shutdown.

Alternatives: Lyse the E. *coli* cells by sonication.

d. Centrifuge lysate at 34,572 ×g (17,000 rpm, rotor radius 107 mm, Thermo Fisher A27-8 x 50 fixed-angle rotor, Thermo Fisher Scientific) for 1 h.
e. Collect supernatant promptly.

Note: If particulate matter persists, filter the solution through a 0.45 μm membrane.

9. Primary Ni-NTA purification.
   a. Pre-equilibrate regenerated Ni-NTA resin with lysis buffer (5 column volumes) and aliquot into 50 mL conical tubes.
   b. Combine clarified lysate with resin matrix, incubating at 4°C with 360° orbital agitation for 30 min.
   c. Load mixture into empty chromatography columns. Collect flow-through after resin sedimentation.
   d. Wash columns with 10–20 column volumes of lysis buffer until baseline UV absorption stabilizes.
   e. Remove nonspecifically bound proteins using 10–20 column volumes of wash buffer. Elute target proteins with elution buffer (2 mL fractions).

Note: Re-equilibrate resin with lysis buffer post-elution.

   f. Monitor protein content via modified Bradford assay: 10 μL eluate + 100 μL dye reagent, terminating collection when chromogenic response plateaus.

Note: CBB Fast Staining Solution (TIANGEN BIOTECH) can be used to roughly evaluate the protein concentration of wash or elution fractions from a gravity column. Add 10 μL of the fractions to 100 μL of dye reagent and observe the depth of the blue color to roughly estimate the protein concentration.

10. SUMO tag cleavage and secondary purification.
   a. Pool protein-containing fractions, quantify total protein concentration, and add Ulp1 protease at 1:1000 (w/w) ratio.

Note: Moderate Ulp1 excess is permissible.

   b. Conduct tandem digestion/dialysis by transferring samples to dialysis tubing (leak-tested, clamp-secured). Perform 4 h dialysis at 4°C with constant stirring using magnetic stirrer.
   c. Re-bind dialyzed sample to fresh Ni-NTA resin (4°C, 360° agitation, 10 min).
   d. Re-chromatograph mixture through columns, collecting flow-through containing purified target protein post-resin sedimentation.
   e. Validate purification efficiency by SDS Polyacrylamide gel electrophoresis(SDS-PAGE) analysis of all fractions.

Note: Analyze 5 μL samples from each purification stage (whole-cell lysate, post-centrifugation supernatant, Ni-NTA affinity chromatography flow-through, wash fractions, and elution fractions) by SDS-PAGE to compare the target protein's relative abundance across stages, assess its purity, and evaluate purification efficiency. Observe sharp-edged bands without smearing or trailing, indicating intact proteins with minimal degradation.

11. Size-exclusion chromatography (SEC).
    a. Concentrate proteins using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore) with a 30 kDa molecular weight cut-off (MWCO). Centrifuge at 1962 ×g (3,000 rpm, rotor radius 195 mm, TX-750 rotor, Thermo Fisher Scientific) for 20 min/cycle at 4°C until volume reaches ~2 mL.

Note: When selecting ultrafiltration concentrators, ensure that the molecular weight cutoff (MWCO) is lower than the molecular weight of the target protein. During concentration, centrifuge at <5,000 rpm (or equivalent ×g force based on rotor specifications) with single-cycle durations ≤30 min to avoid membrane damage. After each cycle, analyze the filtrate (e.g., by SDS-PAGE or Western blot) to confirm the absence of the target protein. If detected, immediately replace the concentrator due to membrane integrity failure.

    b. Conduct buffer exchange via centrifugal filtration to prevent column damage from buffer incompatibility. Troubleshooting 2.
        i. Add 5 mL SEC buffer to the concentrator.
        ii. Perform balanced centrifugation at 1962 ×g (3,000 rpm, rotor radius 195 mm, TX-750 multi-purpose horizontal rotor, Thermo Fisher Scientific) for 20 min at 4°C.
        iii. Repeat 4-5 cycles until final sample volume reaches ~2 mL in SEC buffer.
    c. Aliquot concentrated samples into 1.5 mL microcentrifuge tubes.
    d. Clarify the sample by high-speed centrifugation at 17,000 × *g* (Micro CL17R 21 microcentrifuge, 24 × 1.5 mL fixed-angle rotor, Thermo Fisher Scientific) for 10 min at 4°C. Retain supernatant for subsequent purification.
    e. Select appropriate size-exclusion chromatography (SEC) columns (HiPrep 16/600 Sephacryl S-200 HR or HiPrep 16/300 Sephacryl S-200 HR) based on separation requirements.
    f. Equilibrate SEC columns with 1 column volume (CV) of SEC buffer at 0.5 mL/min flow rate.
    g. Prime injection loops (0.5–5 mL capacity) with SEC buffer through 10 washing cycles.
    h. Load clarified sample using gas-tight syringes, eliminating air bubbles. Match injection volume to loop capacity, then initiate separation in "Inject" mode on AKTA systems.
    i. Collect elution fractions based on UV280 chromatographic profiles: 1 mL/fraction for HiPrep 16/600 columns; 0.5 mL/fraction for HiPrep 16/300 columns.
    j. Analyze column input and SEC eluates by SDS-PAGE to assess purification efficiency.
12. Final concentration and storage.
    a. Concentrate purified proteins to 20 mg/mL using centrifugal filters.

Note: Use Nanodrop (Thermo Fisher Scientific) to determine the OD_{280} value, and calculate the protein concentration using the Beer-Lambert law equation with molar extinction coefficient, which could be obtained from online tools such as ExPASy Protparam tool (https://web.expasy.org/protparam/).

    b. Flash-freeze aliquots in liquid nitrogen and store at −80°C.

Note: Reduce the freeze-thaw cycles to avoid the destabilization of protein and for better repeating of the crystals. Snap-freeze the aliquots every time before putting back to −80°C. Notably, multi-subunit protein complexes exhibit heightened sensitivity to freeze-thaw-induced structural perturbations, which may lead to complex disassembly. For any experiments requiring thawed protein stocks, we rigorously validate structural homogeneity

through analytical size-exclusion chromatography (SEC) to confirm the integrity of the integrity of the target protein. This quality control step ensures that only structurally intact proteins were used for downstream analyses.

Purification of the $E1_{CTD}$-E12 and its mutants

Timing: 1–2 days

This section describes the affinity and size-exclusion chromatography workflow for purifying His-tagged $E1_{CTD}$-E12 complexes and their mutants, omitting tag cleavage steps.(Figures 2B and 2C depict the purification processes of $E1_{CTD}$-E12 and its mutants.)

The N-terminal His tag $E1_{CTD}$-E12 and its mutant constructs are purified using Ni affinity chromatography, followed by size exclusion chromatography (SEC).

The purification process follows the same methodology as described for $E1_{CTD}$ and E12, with the exclusion of step 10 (Ulp1-mediated tag cleavage and subsequent re-purification).

Crystallization and structure determination of E12 and $E1_{CTD}$-E12

Timing: 7–30 days (crystallization)

Timing: 3–7 days (X-ray data collection and structure determination)

This section outlines the vapor diffusion crystallization, X-ray data collection, and molecular replacement-based structure determination for E12 and $E1_{CTD}$-E12 complexes. (Figure 3 shows the procedures for crystal screening and diffraction data collection and analysis of E12 and $E1_{CTD}$-E12.)

13. Initial crystallization screening.
    a. Perform the crystallization screening by using a crystal screening robot of Phoenix (ARI), with commercially available Crystal Screen kit (Hampton) , Index kit (Hampton), Wizard Classic 1/2 kit (Rigaku) and Wizard Classic 3/4 kit (Rigaku).
    b. Conduct primary crystallization trials for E12 and the $E1_{CTD}$-E12 complex (supplemented with 5 mM GTP and 5 mM SAM) using the sitting-drop vapor diffusion method. Troubleshooting 3.
    c. Prepare protein solutions at 15 mg/mL for E12 and 20 mg/mL for the $E1_{CTD}$-E12 complex, with the latter pre-incubated with cofactors on ice for 2 h prior to screening.
    d. Automate crystallization experiments with INTELLI-PLATE 96-well plates (Art Robbins Instruments) under temperature-controlled conditions.
    e. Monitor real-time crystal nucleation and growth through high-resolution imaging via a RockImager system (Formulatrix).
14. Crystallization optimization.
    a. Optimize crystals using a 24-well sitting-drop plate with a grid search strategy to evaluate pH, salt, and precipitant concentrations.
    b. Add 0.7 μL protein solution and 0.7 μL crystallization solution to 400 μL reservoir solution at 18°C via sitting-drop vapor diffusion.
    c. Mix 15 mg/mL of E12 protein with an equal volume of crystallization solution containing 2.0 M ammonium sulfate, 0.1 M CAPS/sodium hydroxide pH 10.5, 0.2 M lithium sulfate, to grow E12 crystals at 18°C.
    d. Mix 20 mg/mL of $E1_{CTD}$-E12 protein supplemented with 5 mM GTP and 5 mM SAM with an equal volume of crystallization solution containing 1600 mM sodium phosphate monobasic/400 mM potassium phosphate dibasic, 100 mM sodium phosphate dibasic/citric acid pH 4.2, to grow $E1_{CTD}$-E12-SAM crystals at 18°C.

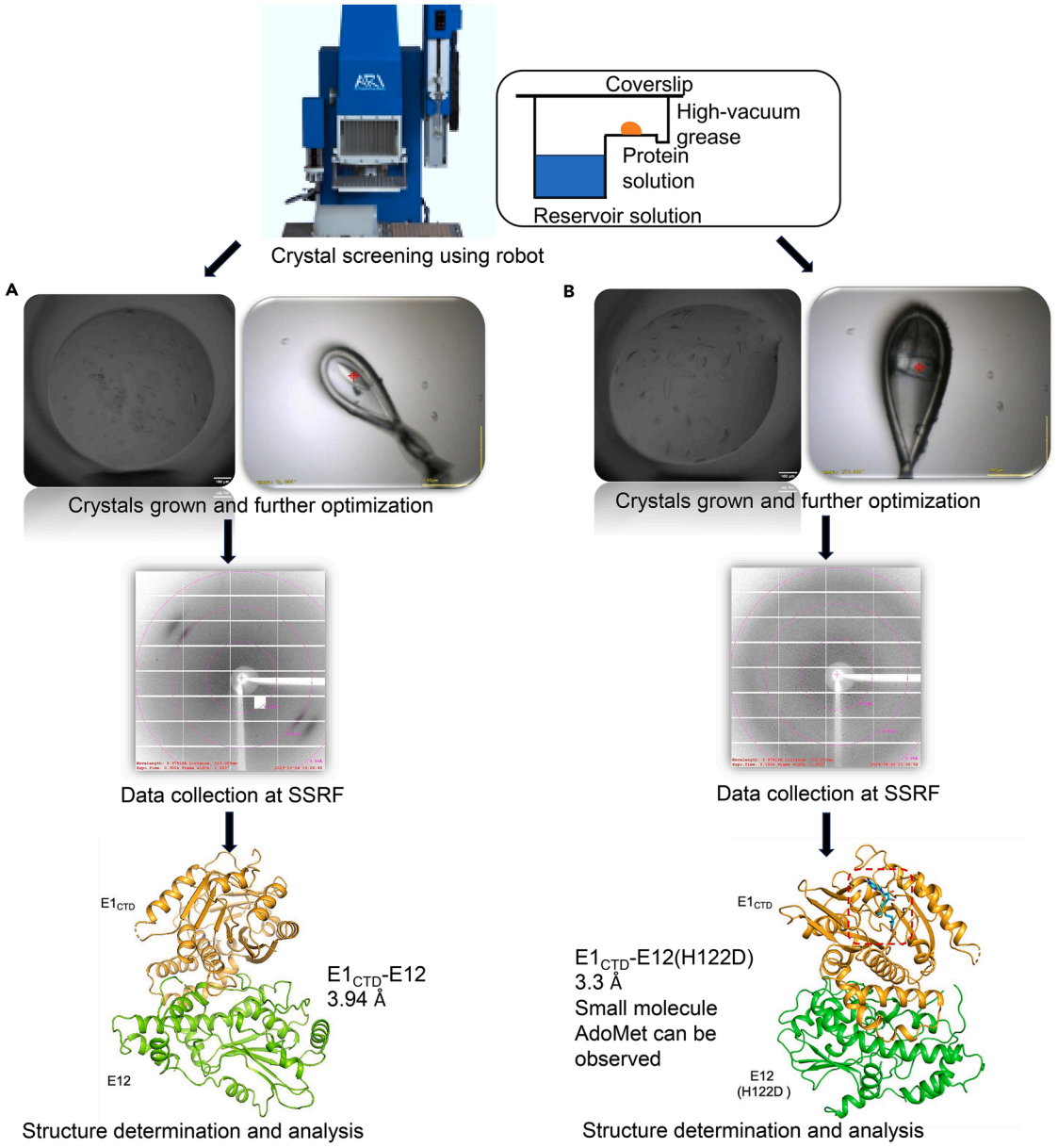

Figure 3. Crystallization and structure determination of E12 and $E1_{CTD}$-E12

(A)$E1_{CTD}$-E12 complex, crystals were grown in a reservoir solution containing 1600 mM sodium phosphate monobasic/400 mM potassium phosphate dibasic, 100 mM sodium phosphate dibasic/citric acid pH 4.2 with a protein concentration of 20 mg/mL. Scale bar, 100 μm.

(B) $E1_{CTD}$-E12(H122D) and AdoMet complex, a mixture of $E1_{CTD}$-E12(H122D) AdoMet (5 mM) and GTP (5 mM) incubated on ice for 1 h before performance of crystallization experiments. $E1_{CTD}$-E12(H122D) and AdoMet complex crystals were grown in a reservoir solution containing 0.1 M Sodium acetate trihydrate pH 4.6, 2.0 M Sodium formate with a protein concentration of 20 mg/mL. Scale bar, 100 μm.

15. Crystal harvesting and cryoprotection.
    a. Harvest crystals using cryoloops appropriate loops under the optical microscope.
    b. Cryoprotect the crystals with 25% glycerol before freezing in liquid nitrogen.
16. X-ray data collection and processing.
    a. Collect X-ray diffraction data at the synchrotron (Shanghai Synchrotron Radiation Facility beam-lines).
    b. Use the XDS program package[4] for indexing, integrating, and scaling of crystallographic data.

17. Molecular replacement and refinement.
    a. Perform crystal structure determinations by molecular replacement.
        i. E12 using AlphaFold2-predicted E12 structure (https://alphafold.ebi.ac.uk) as a search model.
        ii. $E1_{CTD}$-E12 was determined by molecular replacement using the crystal structure of the VACV mRNA methyltransferase structure (PDB: 2VDW).[5]

    Note: Prepare the AlphaFold model with the full-length sequence without truncating flexible loops to preserve structural integrity, and retain all predicted regions (including loop conformations) to ensure biological relevance.

    b. Perform structure refinement with PHENIX.[6]

    Note: Generate initial models in the PHENIX software suite using molecular replacement or ab initio phasing techniques, then perform structural optimization through rigid-body refinement, electron density modification, and all-atom refinement processes.

    c. Perform iterative rounds of the model building using COOT.[4]

    Note: Manually adjust structural models in COOT using electron density maps to perform side-chain optimization, loop reconstruction, ligand/water addition, and stereochemical validation. The software supports interactive refinement with PHENIX for iterative model optimization and is distributed under an open-source license.

    d. Deposit data in Worldwide Protein Data Bank OneDep SYSTEM (https://deposit-2.wwpdb.org/deposition/).

    Note: Timing of crystallization and structure determination may vary depending on the protein and X-ray data quality.

Isothermal titration calorimetry assay

Timing: 1 day

This section describes the measurement of binding affinities between E12 and $E1_{CTD}$, SAM and $E1_{CTD}$, SAM and $E1_{CTD}$-E12 using the Isothermal titration calorimetry (ITC) to determine thermodynamic parameters.(Figure 4 shows the processes and results of the ITC assay.)

18. Sample preparation.
    a. Prepare protein solutions using ITC buffer.

E12 and $E1_{CTD}$	$E1_{CTD}$ 20 μM	E12 100 μM
SAM and $E1_{CTD}$	$E1_{CTD}$ 20 μM	SAM 200 μM
SAM and $E1_{CTD}$-E12	$E1_{CTD}$-E12 20 μM	SAM 200 μM

    Note: Testing buffer-buffer baselines as a negative control measure.

    b. Clarify all samples by centrifugation at 1,370 ×g (Micro CL17R 21 microcentrifuge, 24 × 1.5 mL fixed-angle rotor, Thermo Fisher Scientific) for 10 min at 4°C to eliminate particulates and air bubbles. Troubleshooting 5.
19. Instrument setup and titration experiments.

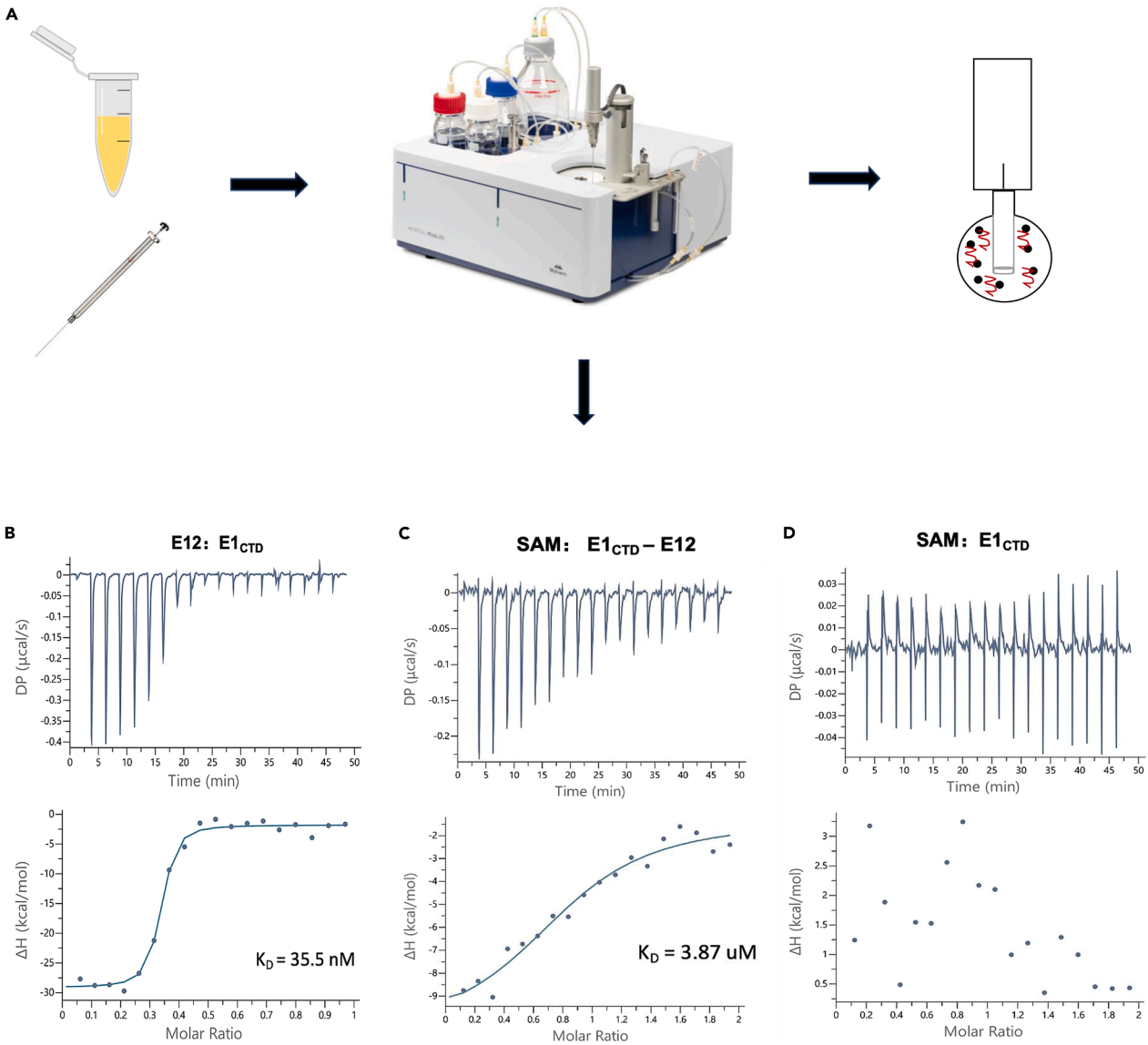

Figure 4. Measure the binding affinity of the interaction between E12 and $E1_{CTD}$, SAM and $E1_{CTD}$, SAM and $E1_{CTD}$-E12 using the isothermal titration calorimetry (ITC)

(A) Preparation of ITC experiment.

(B) Measurements of the binding affinity between $E1_{CTD}$ and E12 by ITC method. The upper panel represents the raw data, revealing the calorimetric response peaks during successive injections of E12 to the $E1_{CTD}$ plot. The lower panel is the best fit of the raw data after subtracting the heat of dilution from the appropriate buffer blank. The solid line in the lower panel shows the best fit of the raw data to the one-set of sites binding model.

(C and D) Measurements of the binding affinity between $E1_{CTD}$, $E1_{CTD}$-E12 complex and SAM by ITC method. The upper panel represents the raw data, revealing the calorimetric response peaks during successive injections of SAM to the $E1_{CTD}$, $E1_{CTD}$-E12 complex plot. The lower panel is the best fit of the raw data after subtracting the heat of dilution from the appropriate buffer blank. The solid line in the lower panel shows the best fit of the raw data to the one-set of sites binding model. Experiments were performed in at least three independent replicates.

a. Prime the sample cell with ITC buffer (Buffer degassing was performed to eliminate air bubbles) through three sequential rinse cycles (300 μL per cycle), followed by complete fluid removal. Troubleshooting 5.
b. Load 300 μL $E1_{CTD}$ into the sample cell using a Hamilton syringe, ensuring bubble-free transfer. Aliquot 70 μL E12 into a PCR tube and position in the instrument's loading station.
c. Execute automated ligand loading using the FillSyringe protocol. Configure titration parameters: 20 injections (2 μL per dose, initial 0.5 μL pulse) at 25°C.

Note: Perform post-experimental system sanitization (SyringePurge and CellClean protocols) with ITC buffer to prevent cross-contamination. Troubleshooting 5.

d. Load 300 μL of $E1_{CTD}$ protein into the sample cell using a Hamilton syringe via bubble-free transfer. Aliquot 70 μL of SAM into a PCR tube and position it at the instrument's designated loading station.
e. Prime the ITC syringe with SAM using the automated syringe priming protocol. Insert the syringe into the sample cell and initiate the titration program with the following parameters: 20 injections (2 μL per cycle, initial 0.5 μL pulse) at 25°C.
f. Transfer 300 μL of the $E1_{CTD}$-E12 complex into the sample cell using identical bubble-free loading techniques. Prepare 70 μL of SAM in a PCR tube and position it at the loading station.
g. Repeat the syringe priming and titration protocol (20 injections, 2 μL/dose, initial 0.5 μL pulse at 25°C) to assess SAM binding to the $E1_{CTD}$-E12 complex.
h. Analyze binding isotherms using MicroCal PEAQ-ITC analysis software. Fit thermodynamic parameters via single-site binding model with baseline correction.

EXPECTED OUTCOMES

The poxvirus mRNA capping enzyme exhibits a unique architectural organization, characterized by multi-domain catalytic modules and a distinct small subunit (E12) configuration compared to orthologous systems. Critical mechanistic questions remain unresolved regarding: Allosteric coordination between functional domains during the capping cascade and spatiotemporal transfer of the mRNA 5′-terminus across catalytic sites. To address these knowledge gaps, we employed integrated structural biology and biochemical approaches. The E12 and $E1_{CTD}$-E12 protein yield obtained after purification typically ranges around 25 mg/L of bacterial culture. The $E1_{CTD}$ protein yield obtained after purification typically ranges around 15 mg/L of bacterial culture. The purity of the E12, $E1_{CTD}$ and $E1_{CTD}$-E12 protein is expected to be > 95% after size exclusion chromatography. X-ray crystallographic determination of E12 and the $E1_{CTD}$-E12 complex (resolved at 2.1–3.9 Å resolution) revealed a conserved SAM-binding pocket with unprecedented mechanistic details. Quantitative isothermal titration calorimetry (ITC) further demonstrated high-affinity intersubunit interactions and identified E12 as an essential SAM-coordinating regulator.

The methodology provides a framework for mechanistic dissection of multi-domain capping enzymes, with applicability to related RNA-processing machineries. The described workflow establishes a reproducible platform for structural and functional characterization of multi-domain enzymatic complexes, with particular relevance to viral RNA-processing machinery studies.

LIMITATIONS

While protein expression and purification remain cornerstone methodologies in biochemical and structural studies, practical implementation often encounters persistent bottlenecks. To address persistent challenges in recombinant protein production, researchers conventionally implement multifaceted optimization strategies, including: using fusion tags to improve solubility, adding protease inhibitors, systematically optimize expression systems by modifying vector design (e.g., promoter strength, codon usage) and selecting compatible host strains, followed by iterative refinement of purification workflows to maximize recovery of functional protein, etc. Monomeric protein expression frequently yields poor solubility or aggregation-prone products; however, coordinated co-expression with cognate interaction partners markedly improves both yield and stability. This protocol presents a novel methodological framework by engineering co-expression vectors to simultaneously produce interacting protein partners. These advanced expression systems serve as robust platforms for multiprotein studies, offering three key advantages: enhanced experimental efficiency, streamlined purification and improved functional integrity.

A persistent challenge in structural biology lies in the inherently stochastic nature of protein crystallization, where success remains empirically determined despite systematic screening approaches. In this study, we successfully determined the crystal structures of E12 and the $E1_{CTD}$-E12 complex, establishing a reproducible framework for crystal growth and diffraction analysis. Initial crystallization trials with $E1_{CTD}$-E12 at suboptimal concentrations (10 mg/mL) in ligand-free conditions failed to produce viable crystals. However, critical optimization through cofactor supplementation (5 mM SAM/GTP) and increased protein concentration (20 mg/mL) enabled successful crystallization, ultimately yielding the ternary $E1_{CTD}$-E12-SAM complex structure.

Limitations of the protocol are mentioned in the troubleshooting section below.

TROUBLESHOOTING

Problem 1

The heterologous expression of the mpox virus-derived mRNA capping enzyme large subunit E1 (encoded by the pSMT3-E1 vector) in *Escherichia coli* resulted in severe solubility limitations, forming predominantly insoluble aggregates (related to steps of "generation of $E1_{CTD}$, $E1_{CTD}$-E12, and $E1_{CTD}$-E12 mutant plasmid constructs").

Potential solution

Based on comprehensive literature analysis, we identified the large subunit E1 of the mpox virus mRNA capping enzyme as a tripartite modular protein comprising: N-terminal RTPase, the middle GTase, and the C-terminal domain (CTD) composed of guanine-N7 MTase. Given the critical role of the MTase domain in small subunit (E12) interaction, we engineered two expression constructs: A truncated variant (pSMT3-$E1_{CTD}$) encoding the isolated MTase domain and a bicistronic co-expression system (pETDuet-$E1_{CTD}$-E12) for the $E1_{CTD}$-E12 complex. These constructs enabled successful production of both $E1_{CTD}$ and the $E1_{CTD}$-E12 complex, which were subsequently subjected to crystallization trials and thermodynamic binding analysis via isothermal titration calorimetry (ITC).

Problem 2

The target protein exhibited pronounced precipitation tendencies during salt gradient reduction, likely due to decreased solubility under low-ionic-strength conditions (related to steps of "purification of $E1_{CTD}$ and E12").

Potential solution

Abrupt reductions in ionic strength (e.g., 500 mM to 100 mM NaCl) frequently destabilize target proteins, leading to nonspecific aggregation and precipitation. To mitigate these effects, we implemented two stabilization approaches: Gradual salt reduction (500 mM-250 mM-100 mM NaCl) improved protein solubility and inclusion of osmolytes (e.g., 10% glycerol or 0.5 M sucrose) preserved native conformation through preferential hydration mechanisms, effectively suppressing precipitation even under low-salt conditions.

Problem 3

Despite extensive crystallization screening, the $E1_{CTD}$-E12 complex failed to produce crystals suitable for X-ray diffraction analysis(related to steps of "crystallization and structure determination of $E1_{CTD}$-E12").

Potential solution

Initial crystallization trials of the $E1_{CTD}$-E12 complex in ligand-free conditions (10 mg/mL) failed to produce detectable crystal nucleation. We increased the concentration of the $E1_{CTD}$-E12 complex to 20 mg/mL in a solution containing 5 mM S-adenosylmethionine (SAM) and 5 mM guanosine triphosphate (GTP) (final concentrations), followed by 1-hour incubation on ice. Subsequent crystal screening under these conditions triggered microcrystalline formation after an extended 4-week

incubation period at 18°C. The limited crystal dimensions necessitate additional optimization strategies to improve diffraction quality.

Problem 4

The $E1_{CTD}$-E12-SAM protein crystals exhibited low resolution(related to steps of "crystallization and structure determination of $E1_{CTD}$-E12").

Potential solution

We resolved the atomic structure of the E12 subunit at 2.1 Å resolution and further elucidated the crystallographic architecture of the $E1_{CTD}$-E12-SAM ternary complex at 3.9 Å resolution. Comparative structural alignment revealed steric constraints between His122 (small subunit) and Lys583 (large subunit) within the capping enzyme assembly, suggesting potential interference with lattice packing. To mitigate this spatial clash, we engineered the $E1_{CTD}$-E12(H122D) mutant using site-directed mutagenesis. Biochemical characterization demonstrated enhanced stability of the mutant complex during multi-step affinity purification. Mix 20 mg/mL of $E1_{CTD}$-E12(H122D) protein supplemented with 5 mM GTP and 5 mM SAM with an equal volume of crystallization solution containing 0.1 M Sodium acetate trihydrate pH 4.6, 2.0 M Sodium formate, to grow $E1_{CTD}$-E12(H122D)-SAM crystals at 18°C. The refined structural model at 3.3 Å resolution enabled unambiguous visualization of the substrate-binding pocket, revealing atomic-level interactions between SAM and conserved residues within the catalytic cleft.

Problem 5

The ITC results of SAM combined with $E1_{CTD}$-E12 were not significant (related to steps of "isothermal titration calorimetry assay").

Potential solution

Reliable isothermal titration calorimetry (ITC) measurements require meticulous attention to the following experimental parameters:

Sample integrity: Use protein aliquots from a single purification batch to ensure consistent concentration and buffer composition.

Instrument performance: Maintain scrupulously clean sample cells and verify baseline stability (DP < 0.1 μcal/s) through water-water control titrations.

Buffer matching: Precisely equilibrate sample and reference buffers to minimize heat artifacts from dilution or mixing.

Optimal concentration: Adjust analyte and ligand concentrations to achieve c-values within the ideal range for accurate thermodynamic parameter determination.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jixi Li (lijixi@fudan.edu.cn).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Jixi Li (lijixi@fudan.edu.cn).

Materials availability

All reagents generated in this study will be made available on reasonable request.

Data and code availability

Coordinates and structure factors have been deposited in the Protein Data Bank under ID codes 8Y2Y, 8Y2Z, and 8ZE4.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.D. and J.L.; methodology and writing, Q.G., A.C., and J.L.; supervision, J.L.; data collection, A.C.; funding acquisition, J.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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