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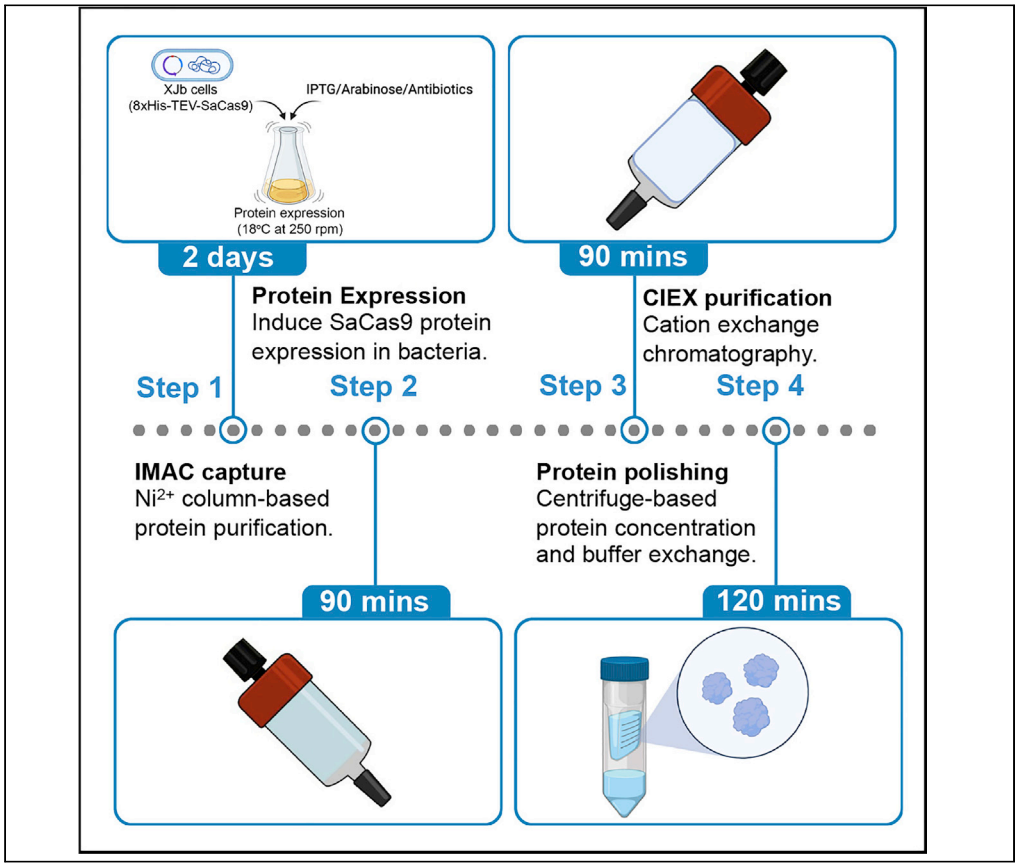
An efficient and cost-effective purification protocol for *Staphylococcus aureus* Cas9 nuclease



Here, we describe a protocol for purifying functional clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) from *Staphylococcus aureus* within 24 h and over 90% purity. SaCas9 purification begins with immobilized metal affinity chromatography, followed by cation exchange chromatography, and ended with centrifugal concentrators. The simplicity, cost-effectiveness, and reproducibility of such protocols will enable general labs to produce a sizable amount of Cas9 proteins, further accelerating CRISPR research.

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

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Highlights

A robust protocol for isolating SaCas9 proteins

Isolate over 10 mg proteins from 4 L of bacterial culture with 90% purity within 24 h

Step-by-step guide for an efficient protein expression and purification

In-gel protein staining and rapid (5 min) protein visualization

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An efficient and cost-effective purification protocol for *Staphylococcus aureus* Cas9 nuclease

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SUMMARY

Here, we describe a protocol for purifying functional clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) from *Staphylococcus aureus* within 24 h and over 90% purity. SaCas9 purification begins with immobilized metal affinity chromatography, followed by cation exchange chromatography, and ended with centrifugal concentrators. The simplicity, cost-effectiveness, and reproducibility of such protocols will enable general labs to produce a sizable amount of Cas9 proteins, further accelerating CRISPR research.

BEFORE YOU BEGIN

Precise editing of genomic DNA remained challenging until the advent of the CRISPR-Cas system. Of the many identified Cas9 proteins, Cas9 from *Staphylococcus aureus* (SaCas9) has emerged as a preferred genetic editing tool, because of its smaller size (1053 amino acids) allowing for an efficient encapsulation into transfecting complexes or transducing viral DNA.[1,2] For example, SaCas9 has been extensively used as a genome editing tool for developing an array of therapeutic strategies investigating human inherited diseases in animal models.[3,4] However, acquiring this protein remains a challenge for many laboratories that are not adequately equipped for protein purification.[5–9] For instance, fast protein liquid chromatography was used for purifying Cas9 proteins in several studies and the equipment requires a hefty initial investment.[6–8] Here, we report an advancement of the purification methods for SaCas9 from bacterial cells. A major advantage of this methodology is to achieve over 90% purity, at large batch sizes (concentrations at 1 mg/L) within a day. Purified SaCas9 can be directly used for *in vitro* applications. The new methodology is superior to the majority of conventional approaches, which rely on expensive infrastructure (*E.g.*, French press, high frequency sonicator, or fast protein liquid chromatography),[5–8] and thereby rendering SaCas9 production more cost-efficient. The average cost for the production of 10 mg of SaCas9 proteins is 86.36 USD (Table 1), significantly lesser than several commercial sources, without compromising the quality of the enzyme itself. Other advantages of this protocol

Table 1. Tabulated consumable cost for the production of 8×His-TEV-SaCas9 proteins

	Unit price in USD
HisTrap column (GE, 17524802)	185.00
HiTrap column (GE, 17115401)	82.40
Amicon Ultra Centrifugal Filter Unit (Sigma, UFC901024)	332.50
LB broth (Bioshop Canada, LBL405)	68.76
LJB autolysis cells (Zymo Research Corporation, 50-444-646)	21.90
PES Bottle Top Filters (Fisher Scientifics, FB12566511)	73.00
Miscellaneous (Salt, glass bottles, carbenicillin, arabinose, etc)	100.00
Total cost for 100 mg of 8×His-TEV-SaCas9 proteins	863.56
Cost per 10 mg of 8×His-TEV-SaCas9 proteins	86.36

On average, it costs 86.36 USD for the production of 10 mg proteins.

include completion of protein purification within 8 h. The setup of the protocol is illustrated in Figure 1.

Institutional permissions

Not applicable.

Prepare SDS-PAGE gels for a rapid in-gel protein visualization

Timing: 30 min

1. Prepare 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel (SDS-PAGE) with 2, 2, 2-Trichloroethanol (TCE) staining agent.
   a. For the running phase, mix 4.0 mL of ddH_2O, 3.3 mL of 30% acrylamide mix, 2.5 mL of 1.5 M Tris-HCl (pH 8.8), 200 μL of TCE (100%) solution, 100 μL of 10% SDS, 100 μL of 10% ammonium persulfate (APS), and 4 μL Tetramethylethylenediamine (TEMED) and transfer a cast mode.
   b. Overlay the gel with 400 μL ddH_2O.
   c. Remove ddH_2O once the gel polymerizes completely.

⚠ CRITICAL: TCE is not readily soluble in the polyacrylamide mixture and should be fully dissolved in ddH_2O with vigorous shaking before adding other ingredients.

   d. For the stacking phase, mix 3.4 mL ddH_2O, 830 μL 30% acrylamide mix, 1.0 M Tris-HCl (pH 6.8), 50 μL of 10% SDS, 50 μL of 10% APS, and 5 μL TEMED. Transfer solution on top of the gel and insert a gel comb. Wait until the gel is fully polymerized.

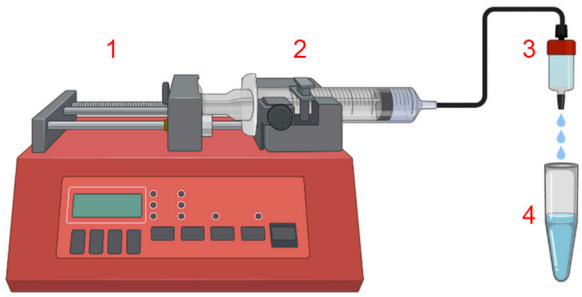

Figure 1. Schematic illustration for protein purification setup

A 50-mL syringe (item # 2) is included for holding protein samples or buffer. The delivery of samples at a consistent speed is controlled by a syringe pump (item # 1). Protein loading, exchange, and elution are completed within columns (item # 3). Eluted proteins in fractions (item # 4) can then be analyzed by rapid in-gel imaging.

⚠ CRITICAL: SDS-PAGE gel can be prepared a week ahead of experiments, provided that it is properly stored in a 4°C fridge.

Prepare LB broth (Lennox) and terrific broth

Timing: 90 min

2. Completely dissolve LB broth (Lennox) and Terrific broth base in ddH_2O as per manufacturer's instruction.
3. Autoclave solution and set aside to cool off before usage.

⚠ CRITICAL: Broth can be stored at 4°C for 6 months. Look for turbidity or precipitation as a sign of contamination.

Prepare LB broth (Lennox) agar plates

Timing: 1 day

4. Completely dissolve LB broth (Lennox) agar base in ddH_2O as per manufacturer's instruction.
5. Autoclave solution to dissolve agar.
6. Cool off LB agar broth to 40°C in water bath. Add carbenicillin to the final concentration of 100 μg/mL and mix thoroughly.

Note: Be very gentle when mixing carbenicillin. Air dissolved in the agar solution can lead to trapped air bubbles.

Note: Other competent cells encoding λ lysozymes can also be used for this protocol.

7. Transfer 20 mL LB agar solution to a petri-dish. Leave agar plates in a tissue culture hood overnight.

⚠ CRITICAL: This step assists in moisture removal in a sterile environment. Do not turn on tissue culture hood.

8. Store agar dishes to a 4°C fridge.

⚠ CRITICAL: Agar plates are stable in fridges for up to 2 months.

Prepare gRNA and template DNA for Cas9 activity assessment

Timing: 1 day

9. Digest pX601 vector with BsaI enzymes by mixing the following reagents and incubate at 37°C for 30 min.

3 μg pX601.
2 μL NEB CutSmart® buffer.
1 μL BsaI enzyme (20 units/μL).
ddH_2O up to 20 μL of volume.

a. Heat-inactivate enzymes at 65°C for 20 min.

10. Subclone double stranded DNA encoding gRNA (5′-CAC CAT GAA ACC ATG GCA AGT AAG-3′, Fwd. primer; 5′-CTT ACT TGC CAT GGT TTC ATC AAA-3′, Rev. primer) against the 55th intronic (in55) of mouse dystrophin gene into pX601 vector via ligation.
    a. Selection of this gRNA was previously described.[10]
    b. Mix the following ingredients and incubate at 16°C overnight.

15 μL ddH_2O.
1 μL BsaI-digested pX601 (0.02 pmol/μL).
1 μL double stranded DNA encoding gRNA (0.02 pmol/μL).
2 μL T4 DNA ligase buffer.
1 μL T4 DNA ligase (20 units/μL).

11. Transform ligated products in DH5′a competent cells (other competent cells, including XL1-blue and DH10B can be used instead).
    a. Transfer 1 μL ligated DNA solution to a 50 μL DH5 α competent cells and incubate on ice for 30 min.
    b. Heat shock bacteria at 42°C for 45 s and back on ice for 2 min.
    c. Add 950 μL antibiotic-free LB broth and inoculate in a bacterial incubator at 37°C and 250 rpm for 1 h.
    d. Transfer 100 μL to carbenicillin-resist agar plate (final carbenicillin concentration on agar plate is 100 μg/mL), spread out bacteria with sterilized glass beads.
        i. Incubate the agar plate upside down overnight in a 37°C incubator.
12. Pick bacterial colonies.
    a. Transfer 3 mL LB broth, supplemented with 100 μg/mL ampicillin, to each of 8 inoculation tubes.
    b. Pick a bacterial colony by dabbing each colony with a sterilized p200 pipette tip, which is dropped into an inoculation tube.
    c. Inoculate bacteria in a bacterial shaker overnight at 37°C/250 rpm.
13. Extract DNA plasmids via QIAprep Spin Miniprep kit according to the manufacturer's instruction.
    a. Confirm the identity of each plasmid by Sanger Sequencing.
    b. Repeat steps 11–13 until a successful clone is obtained).

Note: Purified template DNA (DNA optical densitometry ratio (260/280 nm is 1.7–1.9) can be stored at −20°C for short term (2 weeks) storage. Alternatively, DNA can be stored at −80°C for long term (> 1 month) storage.

14. For generating gRNA, the In55 gRNA-encoding DNA is amplified by Q5 high-fidelity polymerase with the following reagents and cycling parameters.
    a. Reagent mix.

PCR reagents	Final concentration	Amount
Q5 High-Fidelity 2× Master mix	1×	25 μL
10 μM Fwd. primer (5′-TAA TAC GAC TCA CTA TAG GGA TGA AAC CAT GGC AAG TAA G-3′)	0.5 μM	1 μL
10 μM Rev. Primer (5′-AAA ATC TCG CCA ACA AGT TG-3′)	0.5 μM	1 μL
Purified template DNA (pX601-in55 plasmid, 1 ng/μL)	2 ng	2 μL
ddH_2O		21 μL
Total volume		50 μL

b. Cycling parameters.

Steps	Temperature	Time	Cycles
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	30
Annealing	52°C	30 s	
Extension	72°C	20 s	
Final extension	72°C	2 min	1
Hold	4°C	Indefinitely	

15. Purify PCR amplicons via the QIA quick PCR purification kit according to the manufacturer's instruction.
16. Transcribe gRNA from purified amplicon with MEGAshortscript T7 transcription kit and purify gRNA with RNEasy Mini kit according to the manufacturer's instructions.

Note: Purified gRNA (RNA optical densitometry ratio (260/280 nm) is greater than 1.9) can be stored at −20°C for short term (2 weeks) storage or at −80°C for long term (> 1 month) storage.

Note: RNA is labile and sensitive to ambient temperature. It is imperative to leave all RNA samples on ice at all times. To avoid RNase contamination, gloves should be worn, and filter pipet tips should be used. RNaseZap wipes should be applied for cleaning benches and equipment before work.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli DH5α competent cells	Invitrogen	Cat# 18265017
XJb (DE3) autolysis competent cells (F-ompT hsdSB(rB- mB-) gal dcm ΔaraB:: λ, cat (CmR), λ (DE3)	ZYMO Research	Cat# T3051
Recombinant DNA		
8×His-TEV-SaCas9 expression plasmid (The plasmid map is displayed in Figure 2).	Homemade	The plasmid will be available upon request.
pX601-AAV-CMV::NLS-SaCas9-NLS-3×HA-bGHpA;U6::BsaI-sgRNA	Addgene	Cat# 61591
Chemicals and recombinant proteins		
Terrific broth	BioShop	Cat# TER409.1
LB (Lennox) broth	BioShop	Cat# LBL405.1
Carbenicillin	BioShop	Cat# CAR544.1
Isopropyl-b-D-thiogalactopyranoside (IPTG)	BioShop	Cat# IPT001.5
L-arabinose	BioShop	Cat# ARB220.50
Magnesium chloride anhydrous	Sigma-Aldrich	Cat# M8266
Dithiothreitol (DTT)	BioShop	Cat# DTT001.10.1
Imidazole	BioShop	Cat# IMD510.250
Phenylmethylsulfonyl fluoride (PMSF)	BioShop	Cat# PMS123.50
N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid), 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)	Sigma-Aldrich	Cat# H3375
Potassium chloride	Sigma-Aldrich	Cat# P3811
Sodium chloride	Sigma-Aldrich	Cat# S9888
Glycerol	Sigma-Aldrich	Cat# G5516
Benzonase	Sigma-Aldrich	Cat# E1014
30% acrylamide/bis-acrylamide solution	Bio-Rad	Cat# 1610158
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	Cat# T9281

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	Cat# L3771
Ammonium persulfate	Sigma-Aldrich	Cat# A3678
Trizma	Sigma-Aldrich	Cat# T1503
2,2,2-Trichloroethanol (TCE)	Sigma-Aldrich	Cat# T54801
Recombinant shrimp alkaline phosphatase	New England Biolabs	Cat# M0371S
Oligonucleotides		
DMDs Intron 51 gRNA Fwd. primer (5′-CAC CAT GAA ACC ATG GCA AGT AAG-3′)	This paper	
DMDs Intron 51 gRNA Fwd. primer (5′-CTT ACT TGC CAT GGT TTC ATC AAA-3′)	This paper	
In55 amplification Fwd. primer (5′-TAA TAC GAC TCA CTA TAG GGA TGA AAC CAT GGC AAG TAA G-3′)	This paper	
In55 amplification Rev. primer 5′-AAA ATC TCG CCA ACA AGT TG-3′	This paper	
Other		
Amicon Ultra-15 centrifugal filter units	Sigma-Aldrich	Cat# UFC910008
PES bottle top filters	Fisher Scientifics	Cat# FB12566511
HisTrap high performance columns	Cytiva	Cat# 29-0510-21
HiTrap SP high performance columns	Cytiva	Cat# 29-0513-24
Millex GF syringe filter (0.22 µm size)	Sigma-Aldrich	Cat# SLGPR33RS
QIA prep Spin Miniprep Kit	Qiagen	Cat# 27104
Q5 High-Fidelity 2× Master Mix	New England Biolabs	Cat# M0492S
QIA quick PCR purification kit	Qiagen	Cat# 28104
MEGAshortscript T7 transcription kit	Thermo Fisher Scientific	Cat# AM1354
RNEasy Mini kit	Qiagen	Cat# 74104
Syringe pump	Harvard Apparatus	Cat# MS-ACC-0400
Mini-PROTEAN Tetra Handcast Systems	Bio-Rad	Cat# 1658006FC
PowerPac Basic Power Supply	Bio-Rad	Cat# 1645050
ChemiDoc imager	Bio-Rad	Cat# 17001401
Digital general-purpose water bath	Thermo Fisher Scientific	Cat# 51221046
New Brunswick I26 incubator shaker	Eppendorf	Cat# M1324-0000
Centrifuge	Eppendorf	Cat# 5804R
Rotor	Eppendorf	Cat# FA-45-6-30

MATERIALS AND EQUIPMENT

Buffer A (stored in a 4°C fridge for up to 1 month)

Chemicals	Final concentration
HEPES, pH7.5	20 mM
NaCl	300 mM
Imidazole	25 mM

Buffer B (stored in a 4°C fridge for up to 1 month)

Chemicals	Final concentration
HEPES, pH7.5	20 mM
NaCl	300 mM
Imidazole	250 mM

Buffer C (stored in a 4°C fridge for up to 1 month)

Chemicals	Final concentration
HEPES, pH7.5	20 mM
KCl	200 mM
$MgCl_2$	10 mM

Buffer D (stored in a 4°C fridge for up to 1 month)	
Chemicals	Final concentration
HEPES, pH 7.5	20 mM
KCl	1 M
$MgCl_2$	10 mM

SaCa9 storage buffer (stored in a −20°C freezer for up to 6 months)	
Chemicals	Final concentration
Tris-HCl, pH 7.4	10 mM
NaCl	300 mM
EDTA	0.1 mM
DTT	1 mM
Glycerol (v/v)	50%

SaCas9 digestion buffer (stored in a −20°C freezer for up to 6 months)	
Chemicals	Final concentration
HEPES, pH 7.4	20 mM
KCl	150 mM
Glycerol (v/v)	10%
DTT	1 mM

4× protein loading dye (stored at room temperature for up to 6 months)	
Chemicals	Final concentration
Tris-HCl, pH6.8	200 mM
SDS (w/v)	8%
Bromophenol (w/v)	0.4%
Glycerol (v/v)	40%

1× Electrophoresis running buffer (made fresh for each use)	
Chemicals	Final concentration
Tris-HCl	250 mM
Glycine	2 M
SDS	35 mM

STEP-BY-STEP METHOD DETAILS

Transformation of 8×His-TEV-SaCas9 expression plasmid in XJb (DE3) competent cells

Timing: 2 days (for step 1)

The objective is to transform competent cells with 8×His-TEV-SaCas9 plasmids.

1. Transfer 5 ng of 8×His-TEV-SaCas9 plasmid to one tube of XJb (DE3) competent cells. Mix together plasmid and bacteria by finger flicking. Incubate the mixture on ice for 30 min.

   Note: Too much starting plasmid will result in the crowding of bacterial colonies on agar plates.

   Note: Each aliquot of XJb (DE3) competent cells is 100 μL in volume from the manufacturer.

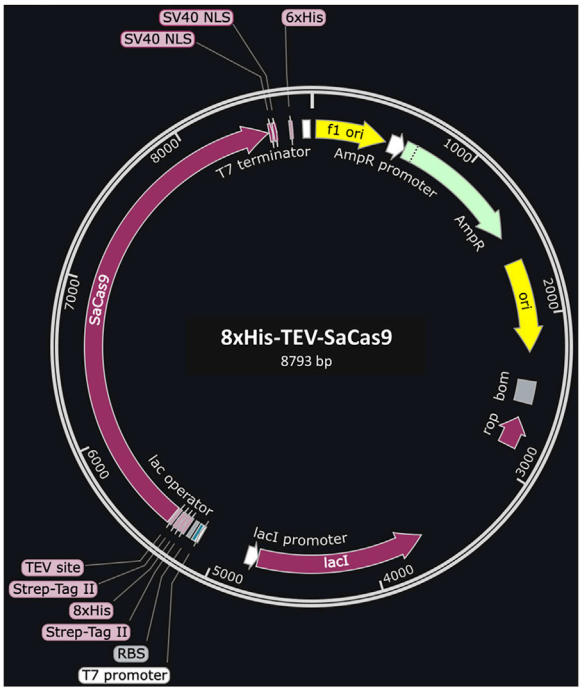

Figure 2. Schematic illustration of 8×His-TEV-SaCas9 plasmid

The expression of 8×His-TEV-SaCas9 is driven by T7 promoter and translation assisted by Shine-Dalgarno sequence (also known as ribosome binding site, RBS). The construct also contains 2 tandem nuclear localization signals from simian virus (SV40 NLS).

2. Heat shock competent cells at 42°C for 1 min and then cool on ice for 2 min.
3. Transfer 950 μL fresh LB broth (Lennox) to competent cells and grow bacteria for 1 h in a bacterial shaker at 37°C and 250 rpm.
4. Evenly distribute 100 μL bacterial culture on a LB (Lennox) agar, supplemented with 100 μg/mL Carbenicillin. Grow bacterial colonies overnight in an incubator at 37°C.

Pause point: The agar plate containing bacterial colonies can be stored at 4°C for no more than 1 month. Each plate needs to be sealed with parafilm to prevent culture contamination.

Inoculating and expending bacterial culture

Timing: 2 days (for step 5)

This step is to expend culture for 8×His-TEV-SaCas9-containing competent cells.

5. Select 2 bacterial colonies from LB (Lennox) agar and grow each colony in 3 mL LB (Lennox) broth, supplemented with 100 μg/mL carbenicillin, in a bacterial shaker overnight at 37°C and 250 rpm. Troubleshooting problem 1.
6. Expand the overnight culture in a 50 mL LB broth (Lennox) culture, supplemented with 100 μg/mL carbenicillin, in a bacterial shaker overnight at 37°C and 250 rpm.

Pause point: Individual bacterial culture can be stored at 4°C for no more than 3 days.

8×His-TEV-SaCas9 protein induction

Timing: 2 days (for step 7)

This step is to induce 8×His-TEV-SaCas9 protein expression with IPTG.

7. Transfer 10 mL bacterial culture from step 6. into 4 × 1 L terrific broth, supplemented with 100 μg/mL carbenicillin. Grow bacteria in a shaker at 37°C and 250 rpm until optical density of 0.6–0.8.

   Note: It usually takes 2–2.5 h to reach the specified optical density.

8. Induce 8×His-TEV-SaCas9 protein expression with 0.5 mM IPTG, 1.5 mM L-arabinose, and 0.5 mM $MgCl_2$ (final concentration). Shake for 30 h at 18°C and 250 rpm.

   Note: L-arabinose and $MgCl_2$ are added for inducing lysozyme expression.

9. Harvest bacteria by centrifugation at 6,000 × *g* for 5 min at 4°C. Decant the supernatant and re-suspend cells in buffer A, supplemented with 1 mM PMSF. Freeze cells at −80°C until protein purification.

   Note: The wet weight of bacterial pellet is typically 1.5–1.7 g per liter of induced bacterial culture.

   Note: The ratio of buffer A and bacteria pellet wet weight is set at 1:10 (v:w). i.e., 10 mL buffer for 1 g bacteria pellet.

   Pause point: Suspended bacteria solution can be stored in a −80°C freezer for no more than 6 months.

Immobilized metal affinity chromatography-assisted enrichment of 8×His-TEV-SaCas9 proteins

Timing: 90 min (for step 10)

This step is for an initial capture of 8×His-TEV-SaCas9 proteins with a Ni^{2+}-NTA column.

10. Lyse cells by freeze and thaw between liquid nitrogen and 37°C water bath 2 times.

    Note: A successful lysis procedure can be observed when liquid viscosity is transformed from runny to an egg white consistency. Troubleshooting problem 2.

11. Remove ribonucleic acids with benzonase at 37°C for 30 min (0.5 μL benzonase (250 unit/μL) per 10 mL bacterial lysate).

    Note: Adding too much benzonase will lead to benzonase contamination and adverse effects in SaCas9 enzymatic analysis.

12. Spin down the insoluble fraction at 15,000 × *g* at 4°C for 30 min.
    a. Filter and degas soluble proteins (soluble fraction) via a 0.22 μm filter unit.
    b. Set aside 100 μL protein lysate in a microtube labeled cell-free lysate. Troubleshooting problem 3.

    Note: All protein fractions and buffers need to be chilled from this point onward.

13. Set up chromatography as demonstrated in Figure 1. Equilibrate a 5-mL HisTrap high performance column with 25 mL ddH_2O, 25 mL buffer B, and 25 mL buffer A (in this order) with a syringe pump at the rate of 2 mL/min.
14. Perform His-tag protein purification by loading degassed samples through HisTrap column via a syringe pump at the rate of 2 mL/min.
    a. Collect unbound proteins in a clean bottle and set aside 100 μL protein lysate in a microtube labeled flow through.

Note: Do not exceed 150 mL of precleared, degassed cell-free lysate per batch purification.

15. Wash off unbound proteins once with 50 mL buffer A and once more with 50 mL of 20% buffer B (40 mL buffer A and 10 mL buffer B). Collect washed off proteins in a clean bottle and set aside 100 μL protein lysate in a microtube labeled wash-off.

Note: Figure 3A shows many non-specific bacteria proteins were effectively washed off with 20% buffer B. 8×His-TEV-SaCas9 protein elution was accompanied with Ni^{2+} ion binding proteins (*E.g.*, ion transporters) that would be systematically removed during ion exchange chromatography.

16. Elute 8×His-TEV-SaCas9 with 100% buffer B in 6 consecutive fractions, 5 mL per fraction. Set aside 100 μL protein lysate from each fraction and label each tube with corresponding fraction numbers.
17. Analyze enriched 8×His-TEV-SaCas9 proteins on a 10% SDS-PAGE gel prepared in section D.
    a. Dilute protein lysates from steps 12, 14, 15, and 16 in 4× loading dye + 100 mM DTT.
    b. In a 10% SDS-PAGE gel, load 20 μL sample mixture to each well. Start electrophoresis at 90 V before the dye front reaches the end of stacking phase (about 10–15 min) and 120 V for the running phase (45–60 min).
18. Remove the polyacrylamide gel from a gel case and soak in ddH_2O for 5 s.
19. Visualizing 8×His-TEV-SaCas9 proteins in a ChemiDoc imager with the following settings.
    a. Image size -> medium. Application -> protein gels -> stain-free gel. Exposure -> gel activation -> 45 s.
    b. Combine fractions that contain 8×His-TEV-SaCas9 proteins and dilute with buffer C by 3 folds. Troubleshooting problem 4.

Cation exchange chromatography for the intermediate purification of 8×His-TEV-SaCas9 proteins

Timing: 90 min (for step 20)

This step works as an intermediate purification of 8×His-TEV-SaCas9 proteins.

20. Equilibrate cation exchange chromatography column with 25 mL ddH_2O, 25 mL buffer D, and 25 mL buffer C (in this order) with a syringe pump, at the rate of 2.0 mL/min.
21. Based on the SDS-PAGE gel results from step 19, combined all protein fractions containing 8×His-TEV-SaCas9 in step 16.
    a. Dilute combined protein lysate with 3× the volume of solution C and filter lysate via a 0.22 μm syringe filter.
    b. Load the equilibrated column with protein lysates via a syringe pump at the rate of 2.0 mL/min.
    c. Collect flow through solution and set aside 100 μL in a microcentrifuge tube for analysis (Flow through fraction).

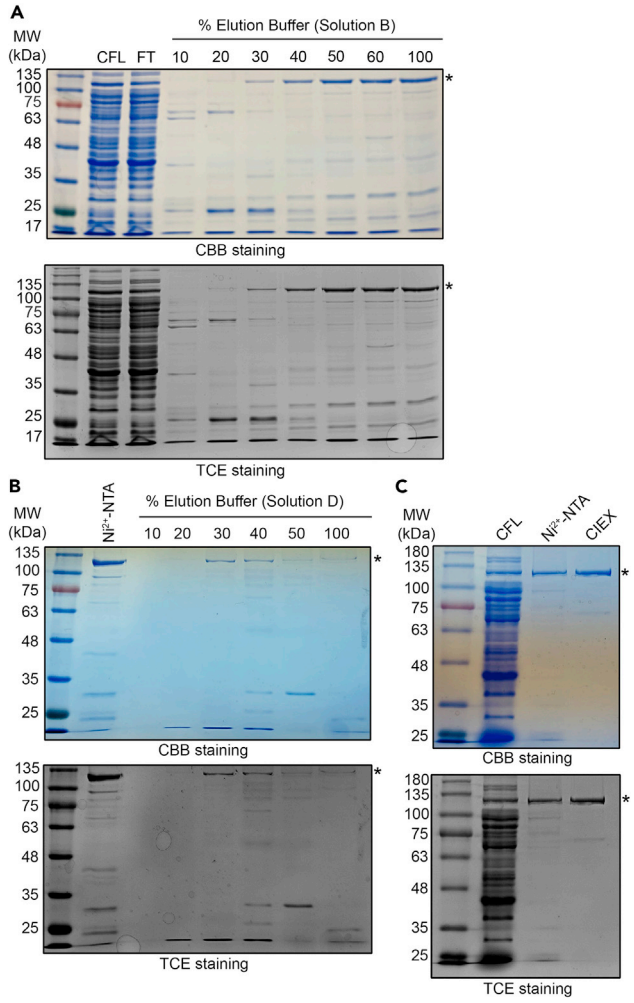

Figure 3. Analyses of Coomassie brilliant blue (CBB) and TCE stained SDS-PAGE gels for purified 8×His-TEV-SaCas9 proteins

(A) Affinity purification of 8×His-TEV-SaCas9. CBB- and TCE-stained SDS-PAGE gels display 8×His-TEV-SaCas9 protein elution with an increasing imidazole gradient (10%–100% solution B) from a Ni^{2+}-NTA column. These empirical results demonstrate that 8×His-TEV-SaCas9 proteins can be enriched in an isocratic fashion – 20% solution B for washing off impurities and protein elution in 30 mL (5 mL per fraction and 6 fractions in total) of 100% buffer B. Top panel, CBB staining; Bottom panel, TCE staining.

(B) Intermediate purification of 8×His-TEV-SaCas9 with CIEX. 8×His-TEV-SaCas9 proteins from Ni^{2+}-NTA-based affinity purification were further fractionated by CIEX. CBB- and TCE-stained SDS-PAGE gels display 8×His-TEV-SaCas9 protein elution with an increasing salt gradient (10%–100% solution D) from a CIEX column. These empirical results demonstrate that 8×His-TEV-SaCas9 proteins can be enriched in an isocratic fashion – 20% solution D for washing off impurities and protein elution in 30 mL (5 mL per fraction and 6 fractions in total) of 30% buffer D. Top panel, CBB staining; Bottom panel, TCE staining.

(C) A representative image of sequentially purified 8×His-TEV-SaCas9 proteins. Total proteins from cell-free lysate, Ni^{2+}-NTA elution, and centrifugal filtration were visualized on a Coomassie brilliant blue-stained SDS-PAGE gel. Top panel, CBB staining; Bottom panel, TCE staining. CFL, cell-free lysate; FT, flow through; *, 8×His-TEV-SaCas9.

22. Wash off unbound proteins once with 50 mL buffer C and once more with 50 mL of 20% buffer D (40 mL buffer C and 10 mL buffer D). Collect wash off solution and set aside 100 μL in a micro-centrifuge tube for analysis (wash off fraction).
23. Elute 8×His-TEV-SaCas9 proteins with 30% buffer D (35 mL buffer C and 15 mL buffer D) in 6 consecutive fractions, 5 mL per fraction. Set aside 100 μL protein lysate from each fraction and label each tube with corresponding fraction numbers.

Note: Figure 2B shows that 30% buffer B eluted mostly 8×His-TEV-SaCas9 with minimal contaminating proteins.

24. Analyze enriched 8×His-TEV-SaCas9 proteins on a 10% SDS-PAGE gel.
    a. Dilute protein lysates from steps 16, 21, 22, and 23 in 4× loading dye + 100 mM DTT.
    b. In a 10% SDS-PAGE gel, load 20 μL sample mixture to each well. Start electrophoresis at 90 V for samples to reach the end of stacking phase (about 10–15 min) and 120 V for the running phase (45–60 min).
25. Remove the polyacrylamide gel from a gel case and soak in ddH_2O for 5 s.
26. Visualizing 8×His-TEV-SaCas9 proteins in a ChemiDoc imager with the following settings. Image size -> medium. Application -> protein gels -> stain-free gel. Exposure -> gel activation -> 45 s. Combine fractions that contain 8×His-TEV-SaCas9 proteins.

Note: The gradual purification process of 8×His-TEV-SaCas9 from crude lysate to final products can be visualized in Figure 3C.

Concentration and buffer exchange of 8×His-TEV-SaCas9 proteins

Timing: 120 min (for step 27)

Purified 8×His-TEV-SaCas9 proteins are concentrated, and buffer exchanged for storage.

27. According to step 26, combined all fractions containing purified 8×His-TEV-SaCas9 proteins.
    a. Filter purified 8×His-TEV-SaCas9 proteins through a 0.22-μm syringe filter. Mix purified His_8-SaCas9 proteins with Cas9 storage buffer in a 1:1 ratio.

Note: All actions from this step onward need to be performed in a sterile laminar flow hood to avoid pathogen contamination if enzymes are used for *in vivo* applications.

28. Equilibrate an Amicon ultra-15 centrifugal filter unit with 10 mL of Cas9 storage buffer. Set aside for 10 min. Centrifuge for 10 min at 3,000 × *g*.
29. Transfer 15 mL of 8×His-TEV-SaCas9 to centrifugal filter unit and centrifuge at 3,000 × *g*, 4°C until 1 mL of solution remains.
    a. Repeat this process until all 8×His-TEV-SaCas9 solution has been filtered.
30. Wash the filter unit with 15 mL of storage buffer and repeat 2 more times.
31. Transfer the retentate containing 8×His-TEV-SaCas9 proteins in the upper unit of a centrifuge filter to a new microtube, labeled purified 8×His-TEV-SaCas9 proteins.
32. Measure protein concentration via Bradford assays and dilute proteins to the final concentration of 5 mg/mL.

Note: The purity and integrity of homemade 8×His-TEV-SaCas9 is comparable to 2 commercially available enzymes as demonstrated in Figure 4A.

Determine 8×His-TEV-SaCas9 enzymatic activity

Timing: 30 min (for step 33)

Test the enzymatic activity of purified 8×His-TEV-SaCas9 proteins.

33. Digestion set up is listed in Table 2.
    a. Mix together digestion ingredients as outlined in Table 2, vortex, spin down, and incubate at 37°C for 60 min.

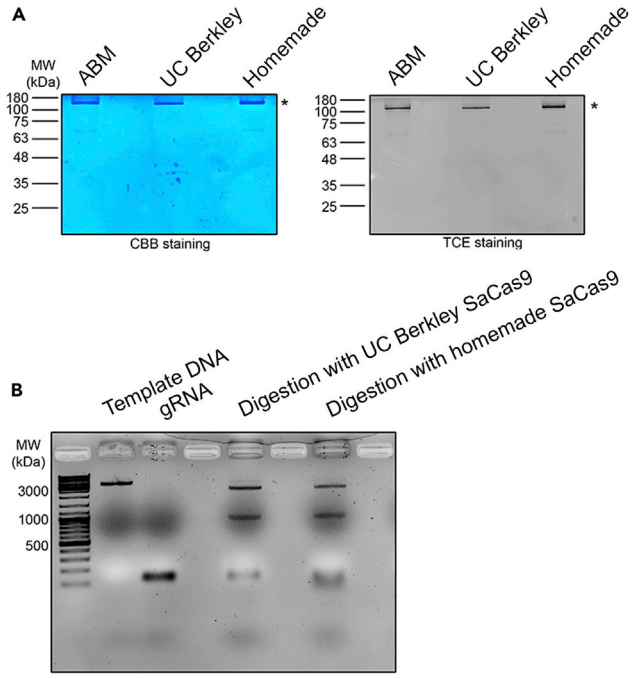

Figure 4. Comparison of homemade 8×His-TEV-SaCas9 to commercial sources

(A) Homemade 8×His-TEV-SaCas9 purification procedure achieves 90% purity, which is comparable to commercial suppliers. The purity of *de novo* 8×His-TEV-SaCas9 proteins (1) was visualized side-by-side with 2 other commercial sources (2 & 3) on either CBB or TCE-stained SDS-PAGE gels. The average cost of 8×His-TEV-SaCas9 proteins purified according to the reported method is 8.64 USD/mg.

(B) Purified 8×His-TEV-SaCas9 efficiently digested double stranded DNA. 8×His-TEV-SaCas9-mediated DNA digestion was analyzed on a 1% agarose gel. A full-length (undigested) template DNA and gRNA were detected in lanes 1 and 2, respectively. A complete digestion of template DNA with commercially supplied 8×His-TEV-SaCas9 was included as a positive control and showed 2 separate fragments (2 and 1 kilobase pairs) in lane 3. A complete digestion of template DNA with homemade 8×His-TEV-SaCas9 was also observed as 2 fragments in lane 4.

Note: Listed in Table 2 includes template DNA only control, gRNA only control, mocked digestion control (template DNA + gRNA), and digestion test (template DNA + gRNA + 8×His-TEV-SaCas9).

34. Heat inactivates enzymes at 65°C for 10 min and assesses digestion results on a 1% agarose gel.

Note: Successful DNA digestion results in 2 DNA fragments (1,000 bps and 3,000 bps) as demonstrated in Figure 4B. Undigested plasmid DNA is 4,000 bps in length.

EXPECTED OUTCOMES

A successful purification procedure should produce 2–4 mg (from 4 L of bacterial culture) of functional, high quality 8×His-TEV-SaCas9 proteins within 24 h. Enriched 8×His-TEV-SaCas9 can be detected as a 135 kDa protein fragment on an SDS-PAGE gel (Figures 3 and 4). The typical yield

Table 2. Experimental setups for testing 8×His-TEV-SaCas9 enzymatic activity

	8×His-TEV-SaCas9 (500 ng/μL)	gRNA (300 ng/μL)	Template DNA (60 ng/μL)	10× reaction buffer (μL)	ddH_2O (μL)
Template DNA only			1	2	17
gRNA only		1		2	17
Mock digestion		1	1	2	16
Digestion	1	1	1	2	15

from 1 L of bacterial culture is 0.5–1.0 mg total proteins at 90% purity (Figure 3C). Purified proteins are comparable to commercial sources in terms of purity and enzymatic function (Figure 4). Routine enzymatic analyses suggest that 500 ng of total proteins are sufficient for digesting DNA Template within 15 min *in vitro* (Figure 4B).

LIMITATIONS

This easy-to-use protocol enables non-biochemistry labs to purify high quality 8×His-TEV-SaCas9 proteins in a sizable quantity for downstream applications. One limitation to the protocol is the use of Bio-Rad ChemiDoc imaging system for visualizing in-gel proteins. In the absence of the machine, protein labeling and protein visualization may not be achievable within 5 min. Alternatively, we recommend Coomassie brilliant blue protein staining, which captures in-gel 8×His-TEV-SaCas9 proteins as demonstrated in *Results*. Another limitation is the scalability for protein production. Each syringe pump generates a set amount of mechanical pressure for consistent liquid delivery. Stacking 2 × 5 mL columns in an end-to-end fashion would create too much resistance for the pump pressure to overcome. As such, protein production needs to be completed in batches (no more than 150 mL filtered and degassed bacterial lysate per batch). Finally, it cannot be ruled out that the presence of both 8×His tag and NLS/TEV sites has no impact on DNA digestion *in vitro*. This is especially true that 8×His tag has some affinities for interacting and aggregating with other proteins if purification procedures are not done properly.

TROUBLESHOOTING

Problem 1

No bacteria growth (step 5).

Potential solution

Incorrect antibiotic concentration is used. Some antibiotics are delivered in the form of effect unit per mass (i.e., UI/mg). Follow the manufacturer's instructions when preparing working antibiotic solutions.

Problem 2

Incomplete bacterial lysis (step 10).

Potential solution

90% bacterial lysis can be achieved by 1 round of freeze and thaw. To achieve > 95% lysis, repeat freeze and thaw step 2 more times.

Problem 3

Difficulty in bottle top filtration (step 12).

Potential solution

Filter units are clogged and need to change often. Each 0.22 μm filter unit is capable of filtering about 50–70 mL bacteria lysate.

Problem 4

8×His-TEV-SaCas9 protein is not detected on SDS-PAGE gels (step 19).

Potential solution

Each Ni^{2+}-NTA column in theory enriches 200 mg of 8×His-TEV-SaCas9 proteins. There are a few reasons to account for reduced protein enrichment. 1. Low quantity of 8×His-TEV-SaCas9 proteins is expressed in bacteria. Go back to protein induction step and ensure IPTG induction has been optimized. 0.5 mM IPTG is a good starting point, but IPTG purity may vary from supplier to supplier. When preparing IPTG stock solution, chemical purity should be considered. In addition, avoid repeated freezing and thawing IPTG. It is a good practice to aliquot working solution. The imidazole

concentration in solution A is too high, reducing 8×His-TEV-SaCas9 protein affinity to the column. Readjust imidazole solution concentration. 3. Ni^{2+}-NTA column is clogged with proteins impurities. Perform rounds of column purging according to manufacturer's instruction before protein purification.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anthony Gramolini, anthony.gramolini@utoronto.ca.

Materials availability

All unique/stable reagents generated in this study are available from the lead contact without restriction. Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anthony Gramolini, anthony.gramolini@utoronto.ca.

Data and code availability

This paper does not report Standardized data types. All data reported in this paper will be shared by the lead contact upon request. No original code was generated in this study.

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AUTHOR CONTRIBUTIONS

A.C.T.T., M.T., M.J.M., and A.O.G. conceived initial ideas for 8× His-TEV-SaCas9 protein purification. A.C.T.T., M.T., A.V., and W.L. performed 8×His-TEV-SaCas9 purification, data acquisition, and analysis. R.M.M., E.I., and R.C. conceived initial ideas for gRNA design and experimentation. S.L.E. and K.L. performed gRNA cloning, *in vitro* transcription, and purification. S.S. performed 8×His-TEV-SaCas9 digestion tests. A.C.T.T., M.T., E.I., J.P.S., and A.O.G. prepared manuscript for submission.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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