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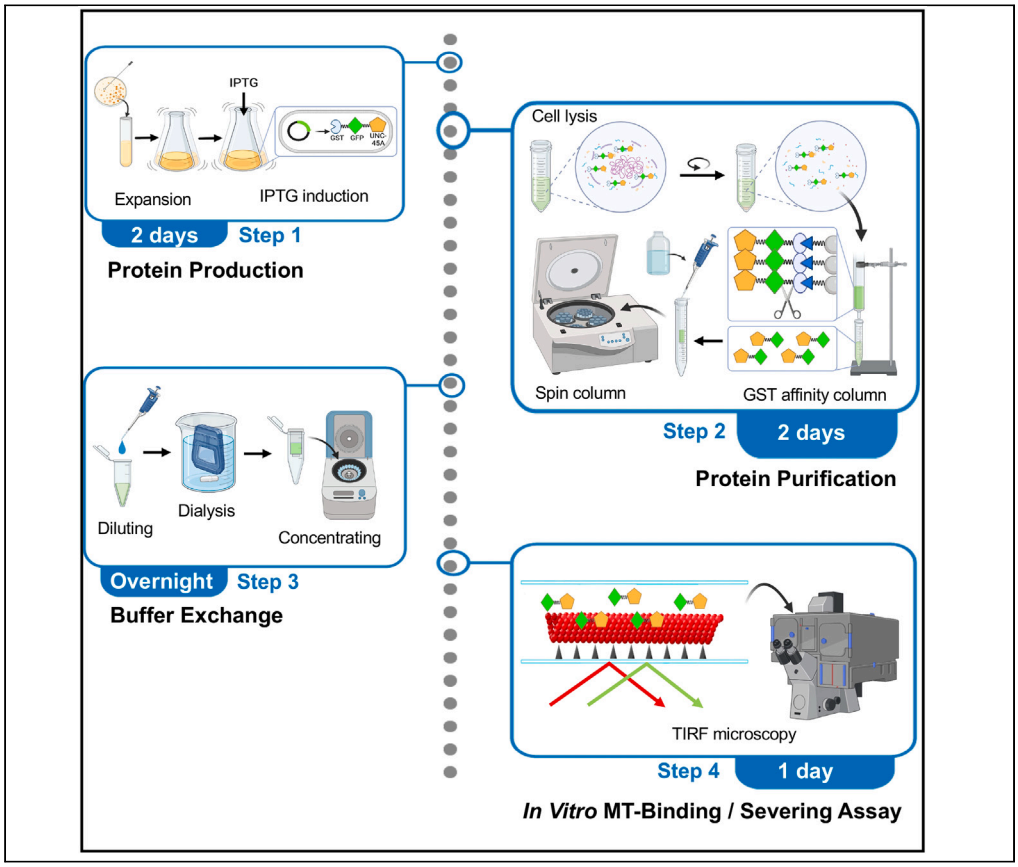
Protocol for purifying biologically active microtubule-severing protein UNC-45A from *E.coli* using GST-affinity and spin columns



Recombinant microtubule (MT)-severing proteins are valuable for studying their mechanisms of action; however, purifying them in an active state is challenging. Here, we provide a protocol to obtain biologically active and highly pure recombinant GFP-UNC-45A, a novel ATP-independent MT-severing protein, from *E. coli*. We describe steps for using GST-affinity and spin columns and detail procedures for assessing the activity of GFP-UNC-45A with *in vitro* MTs along with GFP-katanin as a positive control. The purified proteins can be used for downstream applications to study their functions.

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

Asumi Hoshino, Nimisha Krishnan, Mihir Shetty, Jennifer L. Ross, Martina Bazzaro

hoshi020@umn.edu

Highlights

Instructions for purification of microtubule-severing protein UNC-45A with GFP fusion

Guidance on the preparation of microtubules and TIRF chamber

Steps to evaluate the activity of recombinant protein GFP-UNC-45A using TIRF microscopy

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Protocol for purifying biologically active microtubule-severing protein UNC-45A from *E.coli* using GST-affinity and spin columns

Asumi Hoshino,[1,3,4,*] Nimisha Krishnan,[2] Mihir Shetty,[1] Jennifer L. Ross,[2] and Martina Bazzaro[1]

[1]Masonic Cancer Center and Department of Obstetrics, Gynecology and Women's Health, University of Minnesota, Minneapolis, MN 55455, USA

[2]Physics Department, Syracuse University, Syracuse, NY 13244, USA

[3]Technical contact

[4]Lead contact

*Correspondence: hoshi020@umn.edu
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SUMMARY

Recombinant microtubule (MT)-severing proteins are valuable for studying their mechanisms of action; however, purifying them in an active state is challenging. Here, we provide a protocol to obtain biologically active and highly pure recombinant GFP-UNC-45A, a novel ATP-independent MT-severing protein, from *E. coli*. We describe steps for using GST-affinity and spin columns and detail procedures for assessing the activity of GFP-UNC-45A with *in vitro* MTs along with GFP-katanin as a positive control. The purified proteins can be used for downstream applications to study their functions.

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

BEFORE YOU BEGIN

Obtaining GST-GFP-UNC-45A-expressing *E. coli*

Timing: 2 days

1. Transformation.
   a. Incubate the competent cell/DNA mixture (2–5 μL of pGEX-2TK-GST-muGFP(A206K)-huUNC-45A plasmid, typically 50–100 ng of plasmid, with 50 μL of *E.coli* Rosetta (DE3) pLysS strain on ice for 30 min.
   b. Then place in a hot water bath at 42°C for 60 s, and incubate on ice for 2 min.
   c. Mix with 100 μL of SOC media without antibiotics and incubate at 37°C, 250 rpm for 45 min.
   d. Plate all transformed bacteria and transformed bacteria containing the empty plasmid on agar plates containing 100 mg/mL Ampicillin and 34 mg/mL chloramphenicol at 37°C overnight (typically 16 h).
   e. Check the plates on the next day if bacteria carrying pGEX-GST-GFP-UNC-45A grow colonies and no colonies for bacteria carrying the empty plasmid.

Pause point: After transformation, the agar plate can be stored for approximately one month at 4°C.

Obtaining pMAL-c5X-MBP-GFP-katanin p60-expressing *E. coli*

Timing: 2 days

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2. Transformation.
   a. Thaw purified plasmid (pMAL-c5X-MBP-GFP-katanin p60) on a counter then incubate on ice. Thaw 50 μL of BL21 (DE3) competent cells on ice for about 10 min.
   b. Add ~50–100 ng of plasmid to 50 μL competent cells.
   c. Carefully flick and mix the tube a few times and then incubate on ice for 30 min.
   d. Heat shock at 42°C for 10 s followed by 5 min on ice.
   e. Add 950 μL of SOC media and shake at 37°C, 250 rpm for 60 min.
   f. Mix cells by flicking and inverting the tube, followed by dispensing 25–50 μL onto a pre-warmed LB agar plate with 100 mg/L of carbenicillin.
   g. Spread the cells using a sterilized triangular spreader and incubate at 37°C overnight (12–18 h).
   h. Check the plate on the next day, only bacteria carrying katanin plasmid should grow colonies.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TRITC polyclonal antibody (1:50 dilution)	Invitrogen	A6397; RRID:AB_2536196
Bacterial and virus strains		
E.coli Rosetta (DE3) pLysS	Sigma-Aldrich	70953
BL21(DE3) competent *E. coli*	New England Biolabs	C2527H
Chemicals, peptides, and recombinant proteins		
SOC media	Thermo Scientific	460821
Bacto tryptone	Gibco	211705
Bacto yeast extract	Gibco	212750
DMSO	Fisher Bioreagents	BP231
HEPES	Sigma	H3375
PBS	Corning	21-040-CV
Ampicillin	Sigma	A9393
Chloramphenicol	Fisher Bioreagents	BP904
Carbenicillin	Fisher Bioreagents	BP2648
LB broth (Lennox)	Sigma	L7275
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Fisher Bioreagents	BP1755
PBS	Corning	21-040-CV
Tris-HCl	Millipore	10812846001
NaCl	Fisher Chemical	S271
MgCl	Fluka	63020
DTT	Fisher Scientific	BP172-5
Glycerol	Sigma	G5516
Triton X-100	Sigma	X100
Phenylmethanesulfonylfluoride (PMSF)	Thermo Scientific	36978
cOmplete, EDTA-free protease inhibitor cocktail	Millipore	11873580001
Deoxyribonuclease (DNase) I from bovine pancreas	Sigma	DN25
Thrombin from bovine plasma	Millipore	T4648
ATP disodium salt hydrate	Sigma	A2383
2-mercaptoethanol (BME)	Sigma	M3148
Maltose monohydrate	Sigma	63418-25G
Sodium dodecyl sulfate	Sigma	L3771
Mini-PROTEIN TGX gels	Bio-Rad	4561083
Precision Plus Protein All Blue Standards	Bio-Rad	1610373
Coomassie brilliant blue	Bio-Rad	161-0406
Ethanol	Sigma	459836
Acetic acid	Sigma	A6283
Methanol	Sigma	322415
Bovine serum albumin (BSA)	Fisher Bioreagents	BP1605

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
PIPES	Thermo Scientific	A16090.22
PIPES dipotassium salt	Sigma	P7643
Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)	Sigma	E3889
$MgCl_2$-$6H_2O$	Research Products International	M24000
Mucasol universal detergent	Millipore	Z637181
Ultrapure water	Aqua Solutions, Inc.	W1089
Hydrogen peroxide solution	Sigma	18304
H_2SO_4	Sigma	339741
KOH	Sigma	221473
Trichloroethylene (TCE)	Sigma	251402
Dichlorodimethylsilane (DDS)	Sigma	440272
Tubulin protein (Rhodamine): porcine brain	Cytoskeleton	TL590M
Tubulin protein (>99% pure): porcine brain	Cytoskeleton	T238P
Guanylyl-(alpha, beta)-methylene-diphosphonate (GMPCPP)	Jena Bioscience	NU-405
GTP		
Pluronic F-127	Sigma	P2443
κ-Casein from bovine milk	Millipore	C0406
Glucose oxidase from *Aspergillus*	Millipore	G2133
Catalase from bovine liver	Sigma	C9322
D-glucose	Sigma	G8270
Paclitaxel "Taxol"	Cytoskeleton	TXD01
Critical commercial assays		
Glutathione Sepharose 4B	Cytiva	17075605
Amylose resin	New England Biolabs	E8021S
Bradford protein assay reagent	Thermo Scientific	1856209
Recombinant DNA		
pGEX-2TK-GST-muGFP(A206K)-human UNC-45A	Habicht et al.[1]; Mooneyham et al.[2]; Hoshino et al.[3]	N/A
pMAL-c5X-MBP-GFP-human Katanin p60	Bailey et al.[4]; Lindsay et al.[5]	N/A
Software and algorithms		
ZEN blue edition	Zeiss	https://www.micro-shop.zeiss.com/en/us/softwarefinder/software-categories/zen-blue/
ImageJ Fiji	Schindelin et al.[6]	https://fiji.sc
Excel	Microsoft	https://www.microsoft.com/en-us/microsoft-365/excel
BioRender (graphical abstract)	BioRender	https://biorender.com
Photoshop (graphical abstract)	Adobe	https://www.adobe.com/
Other		
High-speed refrigerated floor centrifuge	Beckman Coulter	N/A
Spectronic 601	Milton Roy	N/A
Floor shaker incubator	GMI	SKU: 8261-30-0003
Emulsiflex c3 homogenizer	AVESTIN	N/A
Digital sonifier	Branson	N/A
Dialysis cassette 3 mL capacity	Thermo Fisher Scientific	66380
Amicon Ultra-0.5 centrifugal filter unit 100 KDa CO	Millipore	UFC510024
Amicon Ultra-4 centrifugal filter unit 100 KDa CO	Millipore	UFC810024
Amicon Ultra-0.5 centrifugal filter unit 30 KDa CO	Millipore	UFC503024
Microscope cover glass 24 × 50 mm no. 1.5	Globe Scientific Inc.	1415-15
Optima TLX ultracentrifuge	Beckman Coulter	N/A
Benchtop centrifuge	Eppendorf	5424
Refrigerated benchtop centrifuge	Eppendorf	5415R

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Axio Observer inverted microscope	Zeiss	N/A
100×, 1.46N/A TIRF objective lens	Zeiss	N/A
1 mL syringe	BD	309628
10 mL syringe	BD	302995
Millex polyethersulfone syringe filter	Sigma	SLGPR33RS
21G × 2 in BD PrecisionGlide needle	BD	305129

MATERIALS AND EQUIPMENT

Luria Bertani (LB) broth with ampicillin and chloramphenicol

- NaCl, 5 g/L.
- Tryptone, 10 g/L.
- Yeast Extract, 5 g/L.
- Ampicillin, 100 mg/mL.
- Chloramphenicol, 34 mg/mL.

PMSF

Prepare a stock solution of 200 mM in DMSO and store the stock solution in aliquots at −20°C.

Protease inhibitor cocktail

Prepare a 25× stock solution by dissolving one tablet into 2 mL of deionized water and store the stock solution in aliquots at −20°C.

Tris-HCl

Prepare 1 M stock at pH 7.5 and store at 4°C.

DTT

Prepare 1 M stock in deionized water and store in aliquots at −20°C.

Thrombin

Reconstitute to 1 unit/1 μL in PBS and store in aliquots at −20°C.

Buffers

UNC-45A Lysis Buffer		
Reagent	Final concentration	Amount
PBS	N/A	25.05 mL
$MgCl_2$	10 mM	300 μL
Glycerol	10%	3 mL
Triton x-100	1%	300 μL
PMSF	1 mM	150 μL
Protease inhibitor cocktail	1×	1.2 mL
Total	N/A	30 mL

Prepared either the same day or a day prior and stored in 4°C. Add PMSF and protease inhibitors and filter-sterilize through a 0.22 μm syringe filter right before proceeding with lysis.

Glutathione Sepharose beads

Before loading proteins, equilibrate 1.5 mL of beads in a gravity column by passing through 7.5 mL of UNC-45A lysis buffer without protease inhibitor and PMSF followed by mixing the beads. Drain

the buffer to let the beads settle, but do not let them dry. Repeat the washing once more. Perform in 4°C cold room.

UNC-45A Wash Buffer		
Reagent	Final concentration	Amount
Tris-HCl pH7.5	20 mM	600 μL
NaCl	500 mM	3 mL
$MgCl_2$	10 mM	300 μL
Glycerol	10%	3 mL
Triton x-100	1%	300 μL
DTT	5 mM	150 μL
Deionized water	N/A	22.65 mL
Total	**N/A**	**30 mL**

Check pH and adjust to 7.5. Store at 4°C for up to a week. Add DTT and filter-sterilize through a 0.22 μm syringe filter directly before use.

UNC-45A Elution Buffer		
Reagent	Final concentration	Amount
Tris-HCl pH7.5	50 mM	1.5 mL
NaCl	500 mM	3 mL
$MgCl_2$	10 mM	300 μL
Glycerol	10%	3 mL
Triton x-100	1%	300 μL
DTT	5 mM	150 μL
Deionized water	N/A	21.75 mL
Total	**N/A**	**30 mL**

Check pH and adjust to 7.5. Store at 4°C for up to a week. Add DTT and filter-sterilize through a 0.22 μm syringe filter directly before use.

5× BRB80 Buffer		
Reagent	Final concentration	Amount
PIPES	160 mM	2.419 g
PIPES dipotassium	240 mM	4.543 g
$MgCl_2$-$6H_2O$	5 mM	0.051 g
EGTA	5 mM	0.095 g
KOH	~128 mM	~0.36 g
Deionized water	N/A	Bring up to 50 mL
Total	**N/A**	**50 mL**

Check pH and adjust to 6.8 by adding KOH. Store at −20°C. Filter-sterilize through a 0.22 μm syringe filter directly before use.

Coomassie Blue Solution		
Reagent	Final concentration	Amount
Coomassie Brilliant Blue	0.2%	2 g
Acetic acid	7.5%	75 mL
Ethanol	50%	500 mL
Deionized water	N/A	Bring up to 1000 mL
Total	**N/A**	**1000 mL**

Coomassie Blue Destaining Solution		
Reagent	Final concentration	Amount
Methanol	50%	500 mL
Acetic acid	10%	10 mL
Deionized water	40%	400 mL
Total	**N/A**	**1000 mL**

Casein

Reconstitute to 20 mg/mL stock in HEPES pH 7.5 by gentle agitation at 4°C overnight and store at 4°C for up to one month. Centrifuge at 20,000 × *g* at 4°C before use.

O_2 scavenger mix

Reconstitute glucose oxidase to 1.10 m/mL and catalase to 0.6 mg/mL (100× O_2 scavenger mix) in 1× BRB80, gently mix, incubate on ice for 5 min, and centrifuge at 20,000 × *g* at 4°C . Filter through 0.22 μm filter, aliquot, snap freeze, and store at −80°C.

UNC-45A Activity Assay Buffer		
Reagent	Final concentration	Amount
Casein	0.2 mg/mL	2 μL
Glucose	20 mM	2 μL
100× O_2 scavenger mix	110 μg/mL glucose oxidase, 60 μg/mL catalase	2 μL
DTT	20 mM	4 μL
5× BRB80	1×	40 μL
GFP-UNC-45A	Desired concentration	N/A
Total	**N/A**	**200 μL**

Prepare on ice and centrifuge at 20,000 × *g* for 10 min right before use.

Luria Bertani (LB) broth with carbenicillin

- NaCl, 5 g/L
- Tryptone, 10 g/L
- Yeast Extract, 5 g/L
- Carbenicillin, 100 mg/L

HEPES

Prepare 1 M stock at pH7.7 and store at 4°C.

ATP

Prepare 100 mM stock and store at −20°C.

Katanin Purification Buffer		
Reagent	Final concentration	Amount
HEPES (pH7.7)	20 mM	1 mL
1 M NaCl	250 mM	12.5 mL
Glycerol	10%	5.5 mL
Protease inhibitor cocktail	1×	2 mL
14.26 M BME	0.5 mM	1.76 μL
ATP	0.25 mM	125 μL
Deionized water	N/A	Bring up to 50 mL
Total	**N/A**	**50 mL**

Prepare on ice on the day of purification and split into 15 mL for lysis buffer, 20 mL for wash buffer, 5 mL for elution buffer, and ~10 mL for amylose resin equilibration. For the elution buffer, add 100 μL of 1 M maltose before use.

Amylose resins

Before loading the bacterial lysate containing MBP-GFP-katanin, wash 2 mL of beads with 2 mL of deionized water by mixing with a pipette and centrifuge at 4000 × *g* for 5 min at 4°C. Carefully discard the supernatant, add 2 mL of katanin purification buffer, mix with a pipette, centrifuge again with the same speed, duration, and temperature. Discard the supernatant and keep the beads on ice.

Taxol

Reconstitute one vile with 100 μL DMSO to make 2 mM stock solution. Make aliquots and store at −20°C.

Pluronic F-127

Dissolve Pluronic F-127 in 1× BRB80 to a final concentration of 10% w/v overnight. Filter the solution through a 0.22 μm syringe filter. Store in aliquots at −20°C.

BSA

Prepare 2 mg/mL stock in PBS and store at 4°C

Katanin Activity Buffer		
Reagent	Final concentration	Amount
100 mM HEPES-HCl pH7.7	20 mM	40 μL
Glycerol	2.5%	2.5 μL
100 mM $MgCl_2$	2 mM	4 μL
ATP	2 mM	4 μL
BSA	0.025 mg/mL	2.5 μL
100× O_2 scavenger mix	110 μg/mL glucose oxidase, 60 μg/mL catalase	2 μL
Glucose	20 mM	2 μL
Pluronic F127	0.05%	1 μL
DTT	10 mM	2 μL
Taxol	10 μM	1 μL
GFP-katanin	Desired concentration	N/A
Deionized water	N/A	Bring up to 200 μL
Total	**N/A**	**200 μL**

Prepare on ice and centrifuge at 20,000 × *g* for 10 min right before use.

Rhodamine-labeled and unlabeled tubulin

Reconstitute 20 μg of rhodamine-labeled tubulin in 2 μL of 1× BRB80. Reconstitute 1 mg of unlabeled tubulin in 100 μL of 1× BRB80. Both of them are 10 mg/mL stock concentration. Make aliquots, snap freeze, and store at −80°C.

GTP

Dissolve 0.52 g of GTP sodium salt hydrate in 7 mL ultrapure water at 25°C. Adjust pH to 7.0 with NaOH. Make aliquots, snap freeze, and store at −80°C.

Zeiss Axio observer inverted microscope

Set up the microscope for TIRF mode at 37°C. Set the exposure time for 561 nm and 488 nm lasers to be 20 ms for the default setting. Set the video recording to be 5 s intervals for 20 min.

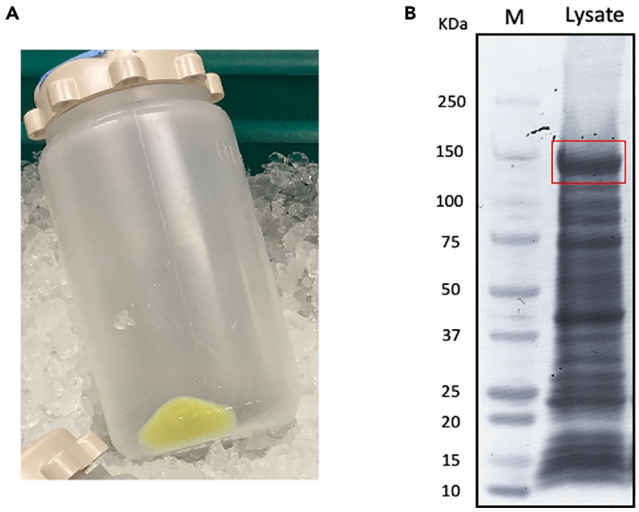

Figure 1. GST-GFP-UNC-45A expression in E.coli

(A) Typical 250 mL bacterial culture pellet after IPTG induction.

(B) 5 μL lysate was solved by 10% SDS-PAGE and stained with Coomassie blue. A band for GST-GFP-UNC-45A is marked by a red square. M, molecular marker.

STEP-BY-STEP METHOD DETAILS

GST-GFP-UNC-45A expression in *E. coli*

Timing: 2 days

In this part of the protocol, *E.coli* cells containing pGEX-2TK-GST-muGFP-UNC-45A plasmid are expanded and GST-GFP-UNC-45A expression is induced by IPTG. The following protocol refers to a 250 mL bacterial culture and can be scaled up or down as needed.

1. Starter culture.
   a. Inoculate 5 mL LB with 100 mg/mL Ampicillin and 34 mg/mL chloramphenicol with a single colony of transformed bacteria in a sterile tube with the lid gently shut.
   b. Incubate overnight (~16 h) at 37°C with shaking at 180 rpm.
2. Large culture and induction of protein expression.
   a. Expand the starter culture by dilution 1/50 into a sterile 500 mL Erlenmeyer flask containing 250 mL of LB with 100 mg/mL ampicillin and 34 mg/mL chloramphenicol.
   b. Incubate for approximately 4 h at 37°C with shaking at 200 rpm until OD_{600}nm = 0.4–0.45 by measuring the OD_{600} at regular intervals.
   c. Lower the temperature of the culture by refrigerating at 4°C for at least 20 min.
   d. Add 500 μM IPTG and incubate for 4.5 h at 16°C with shaking 140 rpm.
   e. Centrifuge the culture for 30 min at 4000 rpm (2500 × g) at 4°C.
   f. Discard the supernatant and freeze the bacterial pellet at −80°C.

Note: After the IPTG induction, the bacterial pellet is usually pale green (Figure 1A).

Pause Point: The bacterial pellet can be stored at −80°C for several months if not intended to proceed with the lysis steps right away.

GFP-UNC-45A purification

Timing: 2 days

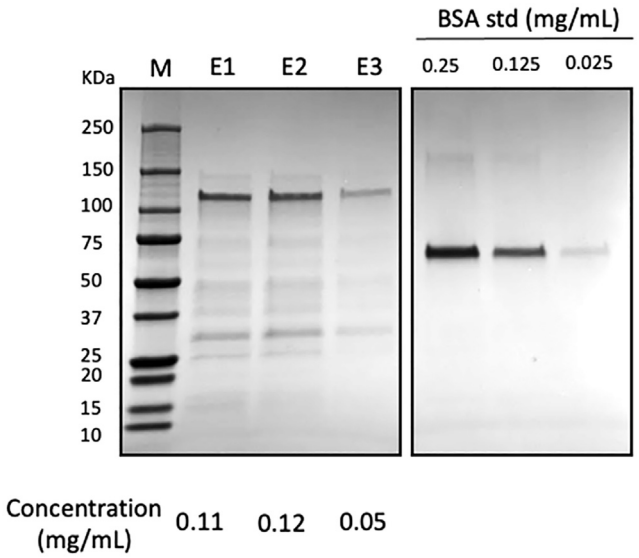

Figure 2. Glutathione Sepharose purification of GFP-UNC-45A

5 μL of each eluted fraction (E1, E2, and E3) was solved by 10% SDS-PAGE along with 5 μL BSA standards and stained with Coomassie blue. The estimated concentration of GFP-UNC-45A in each fraction is shown below each lane. M, molecular marker.

In this part of the protocol, bacterial cells are lysed and GST-GFP-UNC-45A-containing supernatant is subjected to two sequential steps of protein purification to achieve high purity. The first purification step utilizes a GST-affinity column to exploit the specific interaction between glutathione beads and the GST-tagged protein. GFP-UNC-45A is released from glutathione beads by thrombin which cleaves between GST and GFP tags. The eluted GFP-UNC-45A is subjected to a spin column (Amicon Ultra-4 centrifugal filter unit, 100 KDa cutoff) that separates proteins larger than its cutoff size from those smaller.

3. Cell Lysis.

Optional: If the bacterial pellet has been stored in −80°C, thaw the bacterial pellet on ice.

a. Resuspend the pellet in 6 mL of lysis buffer supplemented with PMSF and protease inhibitors.

⚠ CRITICAL: Keep the bacterial resuspension on ice all the time while setting up Emulsiflex homogenizer.

b. Lyse the bacterial cells with Emulxiflex (38 psi) by passing through two passages.

⚠ CRITICAL: GFP-UNC-45A expression in bacterial cells often forms small green insoluble fractions that clog the Emulsiflex. Be sure to remove these fractions before lysing.

c. Supplement the lysate with 0.02 mg/mL DNase I and incubate on ice for 15 min while mixing gently and periodically every 5 min.
d. Supplement the lysate with 400 mM NaCl and incubate on ice for 15 min.
e. Centrifuge the lysate at 10,000 rpm (~12,000 × g) for 1 h at 4°C.
f. Collect the supernatant and supplement it with 5 mM DTT.
g. Keep the supernatant at 4°C.

Pause Point: The supernatant can be snap-frozen and stored at −80°C for several months.

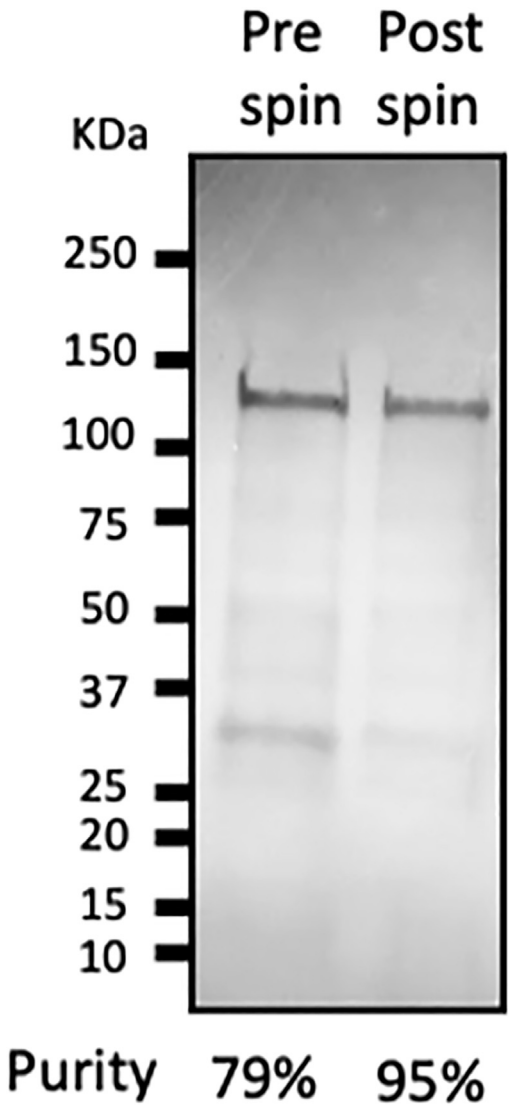

Figure 3. Spin column purification of GFP-UNC-45A

5 μL of pooled GFP-UNC-45A before (Pre-spin, E1 and E2) and after spin column (Post-spin) were solved by 10% SDS-PAGE and stained with Coomassie blue. The estimated purity of GFP-UNC-45A is shown below the lane.

4. GST-affinity chromatography.
   a. Load 6 mL of the supernatant into a pre-equilibrated 1.5 mL glutathione Sepharose gravity column.
      i. Incubate for at least 45 min at 4°C while mixing periodically every 10 min.
      ii. Drain the liquid from the column.
   b. Wash the column with 7.5 mL wash buffer twice.
   c. Add 1.5 mL of elution buffer into the column and 100 units of thrombin and resuspend the beads.
   d. Seal the column securely with parafilm and incubate at 14°C for 2 h at 65 rpm.
   e. Let the beads settle down in the column and drain Elution 1 (E1) at 4°C.
   f. Add 0.75 mL of elution buffer into the column and incubate for 10 min.
   g. Drain elution 2 (E2).
   h. Repeat steps 4f and 4 g for elution 3 (E3).

Note: The volumes can be scaled up or down as per the requirement.

Pause Point: Eluted fractions can be snap-frozen and stored at −80°C for several months.

i. Take 5 μL from each eluted fraction and run an SDS-PAGE along with 5 μL BSA standards.
j. Stain a gel with Coomassie Blue to check protein yields (Figure 2).

Note: To estimate protein concentrations on this gel, see the quantification and statistical analysis section.

5. Spin column.
   a. Rinse the 4 mL spin column by adding ultrapure water to the column and centrifuge at 7,500 × g for 5 min.
   b. Pool eluted fractions containing GFP-UNC-45A (typically E1 and 2).
   c. Spin down the pooled fractions at 16,000 × g for 10 min to remove any aggregates.
   d. Take 5 μL of pre-spin sample from pooled GFP-UNC-45A for SDS-PAGE to check the purity of protein later.
   e. Dilute pooled GFP-UNC-45A with elution buffer by 1/2, and load to the column.
   f. Centrifuge the column at 7,500 × g until the volume of protein becomes half (typically 20 min).
   g. Repeat steps 5d and 5e to keep adding elution buffer to the spin column until the volume of the elution buffer that passes through the spin column is 10 times of the original volume.
   h. Take 5 μL of post-spin sample from pooled GFP-UNC-45A.
   i. Run an SDS-PAGE with pre- and post-spin samples to check protein purity.

⚠ CRITICAL: Do not proceed to the next step unless the protein purity is >95% (Figure 3).

Note: To estimate protein purity on this gel, see the quantification and statistical analysis section.

Pause point: Purified protein can be snap-frozen with elution buffer containing 10% glycerol and stored at −80°C for several months.

Buffer exchange

Timing: Overnight

In this part of the protocol, the elution buffer is exchanged to the protein activity assay buffer (BRB80), which is suitable for experiments with *in-vitro* reconstituted MTs. Throughout the dialysis, GFP-UNC-45A is diluted to minimize protein loss and maintain a pre-active state.

6. Dialysis.
   a. Estimate the protein concentration by either Bradford assay or SDS-PAGE with BSA standards.
   b. Dilute protein with elution buffer to < 0.03 mg/mL.
   c. Activate the 3 mL dialysis cassette by soaking it in BRB80 buffer for 1 min.
   d. Using a syringe and needle, inject GFP-UNC-45A into the cassette.
   e. Place the filled cassette into the BRB80 buffer in a 1 L beaker and slowly stir overnight (typically 16 h).
   f. On the following day, take out GFP-UNC-45A using a syringe and needle.

⚠ CRITICAL: The activity of the protein as well as any downstream experiments needs to be carried out within 48 h of post-dialysis as if the protein is determined active storing the protein in BRB80 buffer for the long term affects the active state of the protein. Even though

protein is diluted in BRB80 at this step, it gradually aggregates and loses its activity over time (typically 48 h).

Pause point: Protein in this state can be stored at 4°C up to 48 h without affecting its activity.

MBP-GFP-katanin expression in *E. coli*

Timing: 2 days

In this part of the protocol, *E. coli* cells containing pMBP-GFP-katanin plasmid are expanded and MBP-GFP-katanin expression is induced by IPTG. The following protocol refers to a 500 mL bacterial culture and can be scaled up or down as needed.

7. Starter culture.
   a. Inoculate 5 mL LB with 100 mg/L of carbenicillin by adding 10–15 single colonies of transformed bacteria in a sterile tube with the lid loosely shut, not tightly.
   b. Set a conical tube at a 35-degree angle in the shaking incubator and incubate with shaking at 250 rpm for 2 h at 37°C until cloudy.
8. Large culture and induction of protein expression.
   a. Expand the starter culture by adding 5 mL starter culture to 500 mL LB media with carbenicillin and shake at 37°C, 250 rpm for about 4 h total.
   b. Measure the OD_{600} at regular intervals until it reaches 0.6–0.9.
   c. Once you achieve the desired OD_{600}, cool down the culture by leaving it at 25°C for 30 min.
   d. Add 1 mM IPTG to induce protein production in cells and shake at 16°C, 250 rpm overnight (at least 16 h).
   e. Centrifuge the bacterial culture at 4°C for 10 min at 5,000 rpm (~3,000 × *g*).
   f. Discard the supernatant and freeze the pellet at −80°C for at least 1 h.

Pause point: The bacterial pellet can be stored at −80°C for several months if not intended to proceed with the lysis steps right away.

MBP-GFP-katanin purification

Timing: 1 day

In this part of the protocol, bacterial cells are lysed and MBP-GFP-katanin is purified by the amylose affinity chromatography which exploit the specific interaction between amylose resins and the MBP-tagged protein. MBP-GFP-katanin is eluted from the resins with maltose, and stored for later MT binding/severing assays.

9. Cell lysis.
   a. Thaw the bacterial pellet on ice and resuspend the pellet in 15 mL of cold katanin lysis buffer by pipetting the buffer onto the pellet and stirring.
   b. Supplement the bacterial resuspension with 0.01 mg/mL DNase.
   c. Sonicate the bacterial resuspension with 20% amplitude for 20 s on and 20 s off to lyse the cell walls and release intracellular contents.

⚠ CRITICAL: Keep the sample on ice to prevent overheating and degradation of sensitive components.

   d. Centrifuge the bacterial lysate for 30 min at 20,000 × *g* at 4°C.
   e. Recover the supernatant and pour it into a sterilized 50 mL beaker and filter it using a syringe and 0.22 μm filter into a clean 50 mL conical tube at 4°C.

10. Amylose affinity chromatography.
    a. Add equilibrated amylose resins into the supernatant and roll/shake at 4°C for 1–2 h.
    b. Collect flow through from the gravity column.
        i. Set up the column on a stand at 4°C.
        ii. Set up a 50 mL conical tube underneath the column.
        iii. Add the supernatant with beads onto the column and collect the flow through in the 50 mL tube placed underneath.

    Note: If the supernatant that flows through the column while the beads are settling is still visibly green, then it likely has a high concentration of GFP-katanin in it. Therefore, this flow through can be saved at −80°C and restart the preparation from step 10a to recover more katanin.

    c. Once the supernatant is almost done flowing through, replace the conical tube underneath with a new conical tube.
    d. Add 15 mL of katanin wash buffer to the column and drain.
    e. Once the wash buffer is almost completely drained from the column, close the column with the stopcock.
    f. Prepare to elute the protein.
        i. Set up and label 5 × 1.5 mL tubes on the top with the elution fraction numbers and mark the 1 mL point on the side of each for easy visualization.
        ii. Place the tubes in an ice bucket underneath the column, starting with tube for fraction 1 directly below the outlet.
        iii. Add 100 μL of 1 M Maltose to 5 mL of katanin elution buffer (20 mM of final concentration).
    g. Add the elution buffer with maltose to the column.
    h. Incubate for 10 min.
    i. Release the stopper and start collecting elution fractions by collecting drips up to the 1 mL line for each tube. Keep the elution fractions on ice.

    ⚠ CRITICAL: Do not let the bead bed dry out at any point during the amylose affinity chromatography.

11. Storing protein.
    a. On a piece of filter paper, draw five circles and label them E1 to E5. Pipette 2 μL of each fraction to the corresponding circle.
    b. Stain the filter paper with Coomassie Brilliant Blue for 30 s, then use destainer to remove the stain for several minutes until distinct dots become visible.

    Note: The intensity of the spots indicates protein concentration, with darker spots representing fractions with higher protein levels. Select the best fractions, typically E2 and E3, for protein storage. This process allows a quick qualitative measure of the protein concentration without needing to run an SDS-PAGE gel.

    Optional: To concentrate the protein further, use a 30 kDa centrifuge filter. Centrifuge the sample at 15,000 × *g* for 3 min. After centrifugation, collect the concentrated protein remaining in the filter and proceed with the next step.

    c. Measure the volume of each elution fraction using a pipette.
    d. Add glycerol to the elution fractions to achieve a final concentration of 25% glycerol in the protein solution, ensuring thorough mixing.
    e. Make appropriately sized, single use aliquots.
    f. Snap-freeze and store in −80°C.

Note: Protein is active for about 1 week after the purification when stored in −80°C.

Note: Ideal product is about 900 μL of 4 μM protein without concentrating the protein, while about 300 μL of 7 μM with concentrating the protein in the best elution fractions using this protocol is achieved.

MT polymerization

Timing: 1–3 h

In this part of the protocol, rhodamine-labeled MTs are polymerize from unlabeled and rhodamine-labeled tubulins for MT binding/severing assays. Two types of MTs; GMPCPP MTs for GFP-UNC-45A and taxol GTP MTs for GFP-katanin are used to see the respective optimum activity.

12. Rhodamine-labeled double-cycle GMPCPP MTs.
    a. Perform the first cycle polymerization.
        i. On ice, mix 2 μL of 10 mg/mL rhodamine-labeled tubulin, 18 μL of 10 mg/mL unlabeled tubulin, and 70.5 μL of 1× BRB80 buffer (20 μM final concentration of total tubulin with 11% labeling).
        ii. Incubate the tubulin mix on ice for 5 min.
        iii. Ultracentrifuge the tubulin mix at 279,000 × g for 10 min at 4°C to remove tubulin aggregates.
        iv. Add 5 μL of 10 mM GMPCPP (0.5 mM final concentration) for 5 min on ice.
        v. Polymerize MTs by incubating at 37°C for 1 h.
        vi. Pellet the MTs by centrifuging at 20,000 × g for 10 min at 25°C.
        vii. Discard the supernatant and gently wash the pellet with warm 1× BRB80 twice.
    b. Perform MT depolymerization.
        i. Resuspend the MT pellet in cold 1× BRB80 such that the final tubulin concentration is ∼2 mg/mL.

Note: The usual recovery is ∼50% of tubulins from the first cycle. Therefore, resuspend the pellet in a half of the original volume of the tubulin mix.

        ii. Incubate the tubulin mix on ice for 30 min with periodically mixing every 10 min by pipette.
    c. Perform the second cycle polymerization.
        i. Add 2.5 μL of 10 mM GMPCPP (0.5 mM final concentration) to the tubulin mix for 5 min on ice.
        ii. Incubate at 37°C for 2 h.
        iii. Pellet the MTs by centrifuging at 20,000 × g for 10 min at 25°C.
        iv. Discard the supernatant and gently wash the pellet with warm 1× BRB80 twice.
        v. Gently resuspend MTs in 800 μL of warm 1× BRB 80 with tip-cut pipette tips.
        vi. Store MTs at 37°C until use.

Pause Point: Double-cycled GMPCPP MTs can be stored at 37°C up to 3 days.

13. Rhodamine-labeled taxol GTP MTs.
    a. On ice, mix 2 μL of 10 mg/mL rhodamine-labeled tubulin, 18 μL of 10 mg/mL unlabeled tubulin, 19 μL of 1× BRB80 buffer (50 μM final concentration of total tubulin with 11% labeling).
    b. Ultracentrifuge the tubulin mix at 279,000 × g for 10 min at 4°C to remove tubulin aggregates.
    c. Add 1 μL of 40 mM GTP and incubate for 20 min at 37°C.
    d. Add 4 μL of 500 μM taxol and incubate for another 20 min at 37°C.

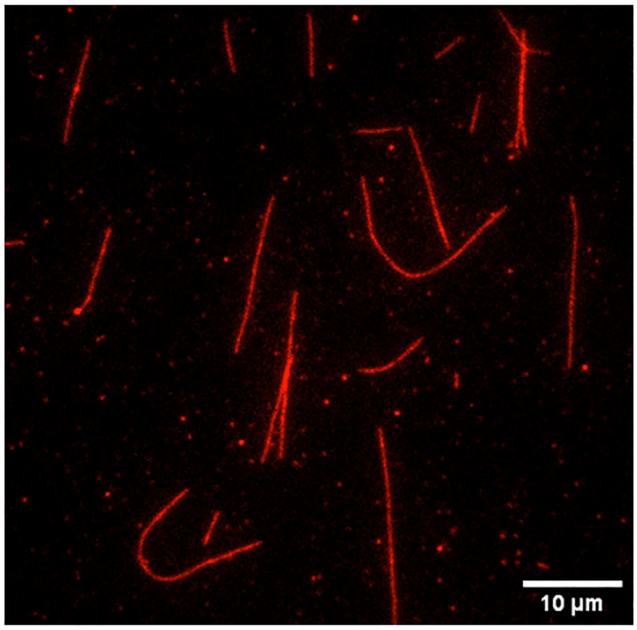

Figure 4. Example MT density for MT severing assay

e. Pellet the MTs by centrifuging at 20,000 × g for 10 min at 25°C.
f. Discard the supernatant and gently resuspend MTs in 800 μL of warm 1× BRB 80 containing 50 μM taxol with tip-cut pipette tips.

Pause point: Taxol GTP MTs can be stored at 37°C up to 5 days.

TIRF chamber assembly and preparation

Timing: 1 day

In this part of the protocol, glass cover slips are cleaned and silanized (introduction of functional groups to the glass surface) to allow immobilization of TRITC antibody on the glass, which later binds to rhodamine-labeled MTs. For glass cleaning and silanization established protocols are used.[7]

14. Glass coverslip preparation.
    a. Place coverslips into racks.
    b. Perform pre-cleaning steps. Perform all steps at 25°C.
        i. Sonicate for 15 min in a soap bath (2% Mucasol, 98% water).
        ii. Rinse in water for 1 min.
        iii. Sequentially bath in acetone for 10 min, ethanol for 10 min, ultrapure water for 1 min.
    c. Perform "Piranha solution" cleaning.
        i. Mix a sufficient volume of 30% H_2O_2 and H_2SO_4 at a ratio of 1:2 in a glass container.

⚠ CRITICAL: Prepare the solution just prior to use since it is only active for ~1 h.

Note: The peroxide should always be added slowly to the acid. If the peroxide concentration is greater than the acid, an explosion could occur.

ii. Transfer the pre-cleaned coverslips directly from water to piranha solution and fully immerse the coverslips.

iii. Bathe the coverslips for 1 h at 60°C.
iv. Transfer the coverslips directly from the piranha solution to three sequential ultrapure water baths for 1 min each at 25°C.

d. Perform KOH treatment. Perform all steps at 25°C.
i. Transfer coverslips to a 0.1 M KOH bath for 15 min.
ii. Transfer the coverslips sequentially through two ultrapure water baths for 1 min for each.
iii. Dry the coverslips completely with in a clean environment.

⚠ CRITICAL: The coverslips need to be completely dry for silanization.

e. Perform glass silanization. Perform all steps at 25°C.
i. Add sufficient TCE to a clean glass container to immerse the cleaned coverslips in the rack.
ii. Add DDS to a final concentration of 0.05% while stirring.
iii. Gently place the coverslips in the TCE/DDS solution and bathe for 1 h.
iv. Transfer the silanized coverslips to three sequential methanol baths placed in an ultrasonic bath for 5, 15, and 30 min.
v. Remove the coverslips from the final methanol bath and dry coverslips completely.
vi. Store the coverslips in a clean sealed container until TIRF chamber assembly.

Pause Point: If the silanized coverslips are not used immediately, they can be stored in 100% ethanol (0.22 μm-filtered) for several months.

15. TIRF chamber preparation.
a. Assemble the TIRF chamber.
i. Cut parafilm into 3 cm × 5 mm.
ii. Stack two parafilm pieces.
iii. Place three stacks of parafilm on a silanized glass coverslip in parallel with 3 mm space in between stacks.
iv. Place a glass slide vertically to the coverslips and parafilms.
v. Heat the stack of glass coverslip, parafilms, and glass slide at 70°C until the parafilms melt to stick with the coverslip and slide.
b. Introduce 40 μL of TRITC antibody (1:50 dilution in BRB80)/channel into the TIRF chamber with a vacuum and incubate for 15 min at 25°C.
c. Introduce 40 μL of 5% Pluronic F127 into the TIRF chamber and incubate for 10 min at room temperature.
d. Wash out the TIRF chamber with 80 μL of BRB80 and store the chamber in a humid container to avoid drying out.
e. On the microscope, focus on the TIRF chamber.
f. Gently introduce 40 μL of MTs to the TIRF chamber with tip-cut pipette tips and check the MT density with MT imaging buffer.

⚠ CRITICAL: Tubulin aggregates from broken MTs decreases UNC-45A activity. Therefore, pipette MTs very gently with cut-tip.

⚠ CRITICAL: Both UNC-45A and katanin activities depend on MT density and too many MTs/ field decreases their activities. Do not exceed 15–20 MTs/field of view (Figure 4).

Optional: If stock MT concentration is too high, excess MTs can be flushed by warm 80 μL 1× BRB80.

g. Adjust TIRF angles so that single MTs are clearly visible.
h. For double-cycled GMPCPP MTs, treat with 10 μM Taxol in BRB80 for 1 h at 37°C.

i. Wash out taxol with 80 μL BRB80.

⚠ CRITICAL: UNC-45A cannot bind to GMPCPP MTs without taxol treatment.[3] Therefore, damage MTs with 10 μM taxol.

GFP-UNC-45A MT binding/severing assay

Timing: 1 day

In this part of the protocol, GFP-UNC-45A is concentrated to trigger its activity, and its activity is assessed by *in vitro* MT-binding/severing assay with TIRF microscopy to ensure its quality and activity. The activity of GFP-UNC-45A is tested in parallel to negative control (no protein added) and positive control (GFP-katanin, the most well-characterized MT severing protein[4,5,8,9]).

16. Preparation of GFP-UNC-45A for activity assay.
    a. Spin down GFP-UNC-45A at 16,000 × g for 10 min at 4°C to remove any aggregates.
    b. Divide dialyzed GFP-UNC-45A into several aliquots typically into 1 mL aliquots.
    c. Rinse the spin column by adding ultrapure water to the column and centrifuge at 14,000 × *g* for 5 min at 4°C.
    d. Concentrate 1 aliquot of GFP-UNC-45A to 0.07–0.1 mg/mL.

    ⚠ CRITICAL: Do not concentrate >0.1 mg/mL, which promotes aggregate formation and causes loss of protein in a filter.

    ⚠ CRITICAL: Do not concentrate <100 μL to avoid pipetting aggregates for activity assay.

    e. Spin down GFP-UNC-45A at 16,000 × g for 10 min at 4°C to remove any aggregates.
    f. Estimate the protein concentration by Bradford assay.
    g. Once the protein concentration is estimated to be in the range of 0.07–0.1 mg/mL, discard this aliquot.

    Note: Bradford assay typically takes 30–40 min. However, the protein must be used immediately after being concentrated, otherwise the substantial amount of protein is lost in aggregates. Therefore, sacrifice the first aliquot for protein concentration estimation.

    h. Take out another dialyzed aliquot and repeat steps 8c–8e.

    ⚠ CRITICAL: Follow the exact same way to concentrate the first aliquot so that the final protein concentration is like the first aliquot.

    i. Immediately introduce concentrated GFP-UNC-45A to the TIRF chamber for MT severing assay (see Step 17d).
17. MT-binding/severing assay.
    a. Quickly warm up katanin activity assay buffer without katanin (negative control).
        i. Flow into the TIRF chamber with taxol GTP MTs.
        ii. Start taking a 20-min video with the TIRF microscope setting described in materials and equipment setup section.

⚠ CRITICAL: Negative control should not break within 20 min video. If MTs are broken within this video, lower the exposure time and the power of lasers, and increase the concentration of oxygen scavenger mix and glucose. Do not start binding/severing assays until no MT breakages happen in the negative control.

b. Quickly warm up the katanin activity assay buffer with the desired concentration of GFP-katanin (positive control).
   i. Flow into a new channel of TIRF chamber with taxol GTP MTs.
   ii. Start taking a 20-min video with the same TIRF microscope setting used for negative control with taxol GTP MTs.
c. Quickly warm upUNC-45A activity assay buffer without UNC-45A (negative control).
   i. Flow in the TIRF chamber with GMPCPP MTs.
   ii. Start taking a 20-min video.
d. Quickly warm up the UNC-45A activity assay buffer with the desired concentration of GFP-UNC-45A (positive control), flow into a new channel of TIRF chamber, and start taking a 20-min video with the same TIRF microscope setting used for negative control.
   i. Flow into a new channel of TIRF chamber with GMPCPP MTs.
   ii. Start taking a 20-min video with the same TIRF microscope setting used for negative control with GMPCPP MTs.

Note: The typical usage is <25 nM of GFP-katanin and 1c00–250 nM GFP-UNC-45A for MT binding assay. Lower concentrations of severing proteins are better to see MT binding since this allows to see clear single molecules binding to MTs. For MT severing, the ideal usage is 25–50 nM GFP-katanin and 250 nM GFP-UNC-45A.

EXPECTED OUTCOMES

After IPTG induction of GST-GFP-UNC-45A (MW = 154 KDa), the bacterial pellet is usually pale green and the lysate has a prominent band at 150 KDa on SDS-PAGE (Figure 1).

After glutathione Sepharose purification, the elution peak is usually observed in E1 and 2, and the majority of green in glutathione beads disappear after E3. Protein yield is approximately 0.125 mg/mL in E1 and 2 and 280 μg of total protein in these fractions (Figure 2).

After the spin column, the purity of the protein is usually 95–98% with 1–2 mL of pooled GFP-UNC-45A at approximately 0.1 mg/mL at the end of this step (Figure 3).

After dialysis and concentrating GFP-UNC-45A, the final purity and concentration are typically 95–98% and 0.07–0.1 mg/mL right before being introduced into the TIRF chamber (Figure 5). 250 mL bacterial culture typically gives us enough GFP-UNC-45A to repeat MT binding/severing assay 3–6 times (Approximately 0.045 mg total protein yield).

For the MT-binding/severing assay, unlike GFP-katanin whose binding increases quickly and covers the entire MT lattice,[4,5] GFP-UNC-45A takes 1–2 min to start binding to MTs in a punctated manner.[1–3] Typically within 5–10 min after the introduction of GFP-UNC-45A, it starts deforming MTs and severing MTs. GFP-UNC-45A needs higher concentrations (ranges from 100–250 nM[1,3]) compared to GFP-katanin (ranges from 25–50 nM[4,5]) to sever MTs (Figure 6).

QUANTIFICATION AND STATISTICAL ANALYSIS

To estimate protein concentrations on Coomassie-stained SDS-PAGE gel, use the following protocol.

1. Open an image of a gel with Fiji, invert the image by clicking Edit > Invert, and open ROI Manager by clicking Analyze > Tools > ROI Manager.
2. Using a rectangle selection tool, draw a rectangle around the band of interest, and click "t" to store selection.
3. Using a rectangle selection tool, draw a rectangle around the adjacent background to the band of the interest, and click "t" to store selection. The area of the rectangle does not need to be identical.

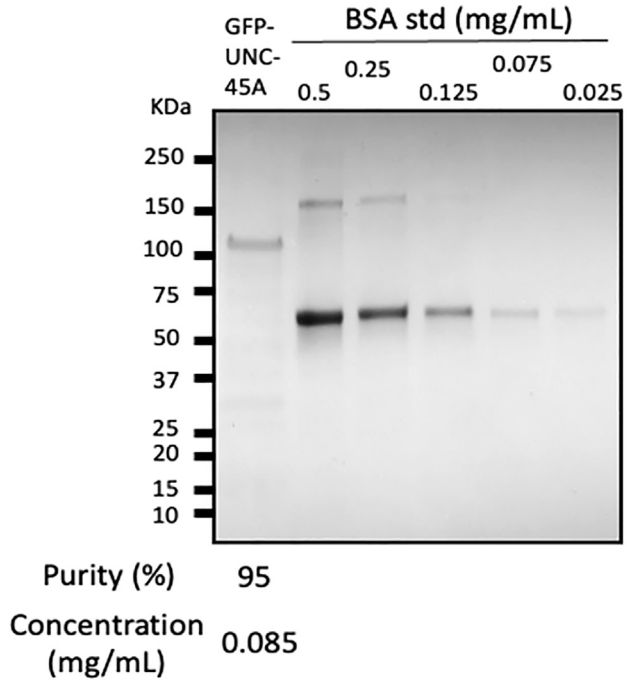

Figure 5. Outcome of GFP-UNC-45A purification

5 μL of GFP-UNC-45A after dialysis and concentrating was solved by 10% SDS-PAGE along with 5 μL BSA standards and stained with Coomassie blue. The estimated concentration and purity of GFP-UNC-45A are shown below the lane.

4. Repeat steps 3 and 4 for BSA standards.
5. Measure the mean gray values in these rectangles by clicking Measure on the ROI manager.
6. In Excel, subtract the mean gray values of the corresponding background from the mean gray values of the band of interest. Repeat this step for BSA standards.
7. Using a scattered plot, plot the concentrations of BSA standards (x-axis) over the background corrected-mean gray values of BSA standards (y-axis), draw a linear best-fit line, and obtain the linear equation.
8. Insert the background corrected mean gray value of the band of interest into the y-value and solve for x to calculate the protein concentration.

To estimate protein purity on Coomassie-stained SDS-PAGE gel, repeat steps 1–6 of the above protocol to obtain background corrected mean gray values for the GFP-UNC-45A band and all the bands below 125 KDa (impurity). Then, the purity is calculated with the following equation:

$$\text{Protein purity (\%)} = \frac{\text{Mean Gray Value}_{\text{GFP}-\text{UNC}-45\text{A}}}{\text{Mean Gray Value}_{\text{GFP}-\text{UNC}-45\text{A}} + \text{Mean Gray Value}_{\text{impurity}}} \times 100$$

Quantify GFP-UNC-45A binding to MTs and MT severing by using Fiji software[6] as described previously.[1,4,5,8,9]

To quantify GFP-UNC-45A binding to MTs, use the following protocol.

9. Open a video of MTs and GFP-UNC-45A with Fiji.
10. On a red channel with MTs, go to the image at 5 min (if the video is taken at 5 s interval, 5 min is 60th frame).
11. Open ORI manager.
12. Select one single straight MT, and draw a rectangle around the MT. Keep the rectangle size just about the same size as the MT.

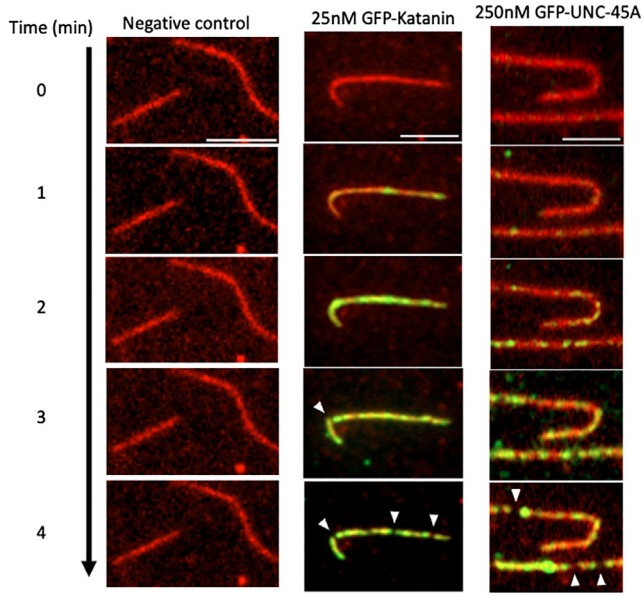

Figure 6. MT severing assay

Rhodamine-labeled MTs (red) visualized by TIRF microscope in the absence of MT-severing protein (negative control), in the presence of 50 nM GFP-katanin (green) (positive control) or 250 nM GFP-UNC-45A (green) at 0 min, 1 min, 2 min, 3 min, and 4 min after the introduction of each protein. White arrowheads indicate MT severing appeared as gaps in the MT lattice. Scale bar, 5 μm.

13. Store MT selection.
14. Move the rectangle selection to adjacent background of the MT, and store background selection. Keep the size of rectangle the same, and avoid UNC-45A aggregates if there is any.
15. Go to green channel with GFP-UNC-45A, and measure integrated density (IntDen) with stored MT and background selections.
16. Subtract GFP-UNC-45A IntDen of the background selection from the MT selection.
17. Measure the length of the MT and divide the corrected GFP-UNC-45A IntDen by the length of the MT to normalize MT length.
18. Select another MT, and repeat steps 4–9 to quantify multiple MTs. Typically quantify includes 50–100 MTs/condition for GFP-UNC-45A binding.

Quantification of MT severing is done with two parameters; MT severing frequency and MT severing percentage.

To quantify MT severing frequency, use the following protocol established by Díaz-Valencia et al.[8,9]

19. Open a video of MTs and GFP-UNC-45A with Fiji.
20. On a red channel with MTs at the time 0 s (1st frame), measure the length of all MTs present in the field of view.
21. Manually count the number of clearly visible severing events along MTs by play the video.
22. Quantify another video by repeating steps 1–3. Typically quantify 3 videos/condition.

Once the number of severing events and MT length are obtained, MT severing frequency is calculated with the following equation:

$$\text{MT severing frequency } (\#/\text{um}^{-s}) = \frac{\text{Number of severing events}}{\left(\frac{\text{Total MT length in the field (um)}}{\text{Video length (s)}}\right)}$$

To quantify percentage MT severing, use the following protocol established by Bailey et al.[4] and Lindsay et al.[5]

23. Open a video of MTs and GFP-UNC-45A with Fiji.
24. Manually count the total number of MTs in the field of view and the number of MTs with at least one severing event during the video.
25. Quantify another video by repeating steps 1 and 2. Ideally quantify 3 videos/condition.

MT severing percentage is calculated with the following equation:

$$\%\ \text{MT severing} = \frac{\text{Number of MTs that undergo at least once during the video}}{\text{Total number of MTs in the field of view}}$$

LIMITATIONS

This protocol was optimized to successfully purify GFP-UNC-45A (wild-type) with greater than 95% purity and was successfully adapted to its mutants, GFP-UNC-45A (N-terminus domain deletion) and (C-terminus domain deletion).[1] However, different conditions for the expression and purification of other mutants may be required and must be established in each case.

Although mouse and human UNC-45A have 94–95% of sequence identity and 96–98% of sequence similarity,[10] further optimization of the protocol may be necessary to purify mouse UNC-45A and UNC-45A from other species.

TROUBLESHOOTING

Problem 1

Protein loss during the IPTG induction results in loss of green in the bacterial pellet. This problem may arise in step-by-step method details step 2.

Potential solution

- Do not go over OD_{600}nm = 0.45 before IPTG induction. Bacterial culture with OD_{600}nm > 0.45 results in loss of protein after IPTG induction.
- Make sure to lower the temperature of the bacterial culture before starting IPTG induction.
- Monitor OD_{600} during the IPTG induction. The best OD_{600} range to stop IPTG induction is 0.7-<0.8. Do not go over $OD_{600} = \geq 0.8$.

Problem 2

Protein forms aggregates during the spin column resulting in protein loss. This problem may arise in step-by-step method details step 5.

Potential solution

Make sure to dilute protein before loading it into the spin column. Do not concentrate the protein > 0.125 mg/mL to avoid the formation of aggregates and protein loss during the spin column.

Problem 3

GFP-UNC-45A solubility decreases in the BRB80 buffer resulting in protein precipitation after the dialysis. This problem may arise in step-by-step method details step 6.

Potential solution

Make sure to dilute protein <0.03 mg/mL before injecting it into the dialysis cassette.

Problem 4

GFP-UNC-45A aggregates before the introduction to the TIRF chamber, which covers its signals on MTs and is unable to observe its binding to MTs. This problem may arise in the step-by-step method details step 8–9.

Potential solution

If the protein is active, concentrating it to 0.07–0.1 mg/mL in BRB80 buffer quickly starts forming new aggregates within a few minutes. Therefore.

- Prepare the TIRF chamber and TIRF microscope ready for the severing assay with GFP-UNC-45A by the time GFP-UNC-45A is concentrated and centrifuged to remove aggregates.
- Make sure to centrifuge GFP-UNC-45A right before introducing to the TIRF chamber.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Asumi Hoshino (hoshi020@umn.edu).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to the technical contact, Asumi Hoshino (hoshi020@umn.edu).

Materials availability

The plasmid pGEX-2TK-GST-muGFP(A206K)-huUNC-45A is available from Martina Bazzaro, mbazzaro@umn.edu, upon reasonable request.

Data and code availability

The published articles include all data generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, methodology, and writing – original draft, A.H. and N.K.; writing – review and editing, M.S. and J.L.R.; funding acquisition, M.B., N.K., and J.L.R.; supervision, M.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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