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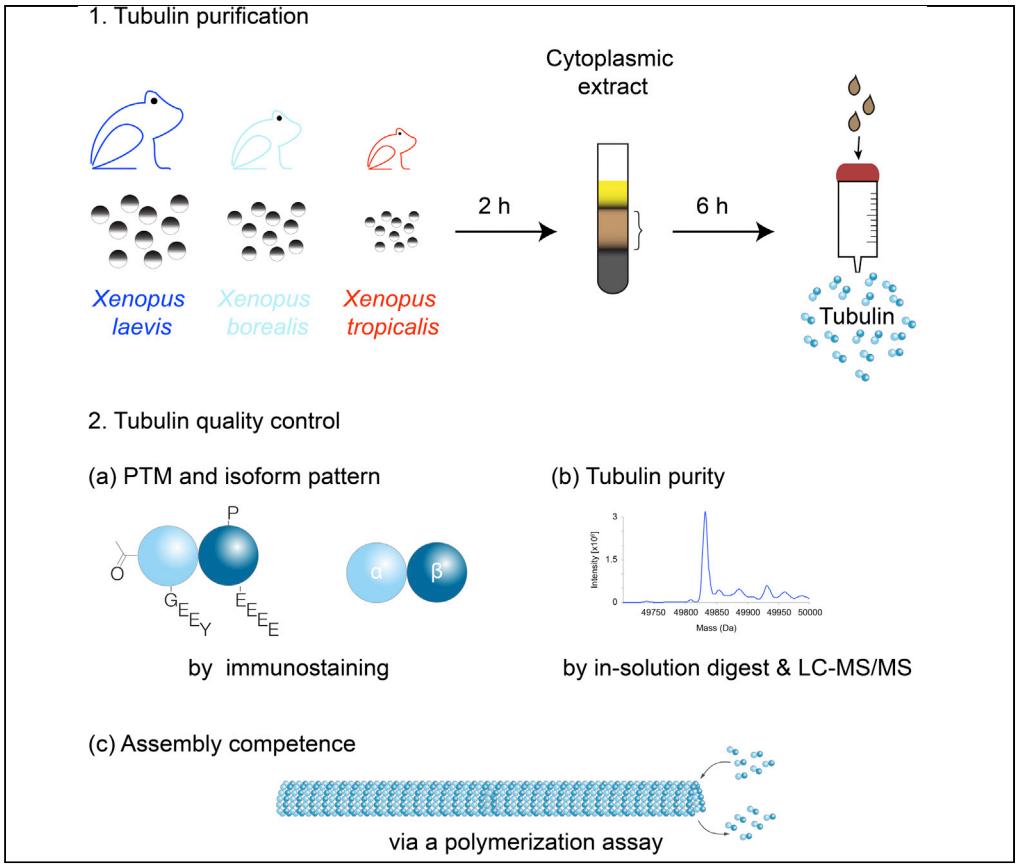
Affinity Purification of Label-free Tubulins from *Xenopus* Egg Extracts



Cytoplasmic extracts from unfertilized *Xenopus* eggs have made important contributions to our understanding of microtubule dynamics, spindle assembly, and scaling. Until recently, these *in vitro* studies relied on the use of heterologous tubulin. This protocol allows for the purification of physiologically relevant *Xenopus* tubulins in milligram yield, which are a complex mixture of isoforms with various post-translational modifications. The protocol is applicable to any cell or tissue of interest.

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HIGHLIGHTS

Purification of native, active tubulin from CSF extracts of different *Xenopus* spp

Tubulin isoform composition and post-translational modifications are preserved

Generally applicable to any cell line or tissue of interest

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Affinity Purification of Label-free Tubulins from *Xenopus* Egg Extracts

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SUMMARY

Cytoplasmic extracts from unfertilized *Xenopus* eggs have made important contributions to our understanding of microtubule dynamics, spindle assembly, and scaling. Until recently, these *in vitro* studies relied on the use of heterologous tubulin. This protocol allows for the purification of physiologically relevant *Xenopus* tubulins in milligram yield, which are a complex mixture of isoforms with various post-translational modifications. The protocol is applicable to any cell or tissue of interest.
For complete details on the use and execution of this protocol, please refer to Hirst et al. (2020).

BEFORE YOU BEGIN

Injection of *Xenopus* Frogs to Induce Egg Laying

Timing: 1–2 h for injections and frog handling (will depend on the number of frogs), 1–5 days prior to tubulin purification

The basic protocol is based on Andrew Murray's original protocol for *Xenopus laevis* (*X.l.*) eggs (Murray 1991) with minor modifications for *X. tropicalis* (*X.t.*) (Brown et al., 2007) and *X. borealis* (*X.b.*) (Kitaoka et al., 2018).

Note: Yields of extract and therefore tubulin will depend on egg quantity and size (Table 1). The eggs of *X.l.* are 1.3±0.05 mm in diameter, similar to *X.b.* eggs with a diameter of 1.2±0.02 mm (Kitaoka et al., 2018). In either case, a batch of eggs from one frog will yield ~1 mL of extract. The eggs of *X.t.*, however, are significantly smaller (0.75±0.02 mm) and will thus yield around 0.35 mL per frog. We recommend starting with at least two-three *X.l.* or *X.b.* frogs and six *X.t.* frogs per experiment.

1. Prepare stock solutions of Pregnant Mare Serum Gonadotrophin (PMSG, 200 U/mL) and Human Chorionic Gonadotrophin (HCG, 2,000 U/mL or 100 U/mL) by dissolving hormones in ultra-pure water. Filter-sterilize the stock solution.

Note: We suggest using fresh aliquots of both hormones. For extended storage, store as single-use aliquots at −20°C for a few months only.

Table 1. Tubulin Purification Yields

Species	Input Egg Extract	Tubulin Yield
Xenopus laevis	3 mL	2.2 mg
Xenopus borealis	2.5 mL	1.4 mg
Xenopus tropicalis	6.5 mL	2.4 mg

2. At least three days before preparing extracts, prime *X.l.* and *X.b.* frogs by injecting them with 100 U and 50 U of PMSG (by diluting the 200 U/mL stock in ultra-pure water), respectively. Primed *X.l.* and *X.b.* can be used for up to 10 days for egg laying after priming. In the case of *X.t.* frogs, prime frogs 16–24 h before extract preparation with 10 U of HCG.

⚠ CRITICAL: The injected volume should not exceed 0.5 mL for *X.l.* and *X.b.*, and 0.1 mL for *X.t.* Gently inject the hormones subcutaneously, dorsally posterior to the lateral line.

⚠ CRITICAL: Do not feed the frogs after priming. Fed frogs often defecate during egg laying, which can impact egg quality and residual debris is often difficult to remove.

Note: For the injections, we suggest using sterile 27-G needles and 1 mL syringes.

3. To induce ovulation, boost primed frogs by HCG injection. For *X.l.*, we recommend injecting 1,000 U of HCG per frog 16–18 h before eggs are needed. For *X.b.*, we suggest using 500 U of HCG per frog 16–24 h before the experiment. For *X.t.*, we recommend a 200 U injection of HCG per frog given 5–6 h before extract preparation.
4. Separate each frog in an individual container. For *X.l.* and *X.b.* frogs, use 1 L of 1× MMR and 0.5× MMR, respectively. For *X.t.*, transfer frogs into containers containing a suitable volume (~0.5 L) of tank water (our frogs are kept in recirculating water with controlled temperature, conductivity of ~1,000 μS and at pH 7.2–7.5).
5. For the egg laying step, transfer containers with frogs to a dark and quiet environment such as an incubator maintained at the appropriate temperature (16°C–18°C for *X.l.*, 16°C for *X.b.* and 25°C–27°C for *X.t.*).

Note: For *X.t.*, temperatures below 23°C have been reported to impact extract quality (Brown et al., 2007).

6. Prepare buffers required for extract prep freshly unless mentioned otherwise. Maintain a 16°C–18°C working environment for extract preparation.

Preparation of the TOG Column

Timing: 2 days

Here, the purification of tubulin takes advantage of the specific binding of TOG domains to tubulin (Widlund et al., 2011, Widlund et al., 2012). The TOG1/2 domain from *Saccharomyces cerevisiae* Stu2 fused to glutathione S-transferase (GST) is expressed as a fusion protein in *Escherichia coli* and conjugated to a N-hydroxysuccinimide ester-activated sepharose column (NHS column), which we then refer to as the TOG column. The TOG domains of *S. cerevisiae* Stu2 bind to every tubulin we tested so far. However, if you unexpectedly will not be able to purify your tubulin of choice, you might consider making a column with the TOG domain of your species of interest. TOG proteins have been found in all eukaryotes (Gard et al., 2004; Al-Bassam and Chang, 2011). The purification of *X.l.* tubulin based on conventional polymerization-depolymerization cycles has been described elsewhere (Groen and Mitchison 2016).

Expression and Purification of GST-TOG1/2

7. Transform the TOG1/2 plasmid (pGEX-6P-1 Stu2 1-590) into BL21(DE3) T1 pRARE cells.
8. Inoculate an overnight culture in LB containing antibiotics (100 μg/mL ampicillin and 15 μg/mL chloramphenicol).

   Note: Make sure to use fresh transformants for the expression.

9. Dilute culture 500-fold into 3 L LB with the same antibiotics and shake at 37°C until the OD_{600} reaches 0.5.

   Note: A total of 3 L expression culture should yield sufficient GST-TOG1/2 (~50–60 mg) to make a 5 mL TOG column.

10. Shift the cultures to 18°C for 1 h.
11. Induce expression with 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubate at 18°C and 220 rpm orbital rotation overnight.
12. Harvest cells by centrifugation in a JLA 8.1000 rotor (or comparable large volume model) for 5 min at 5,000 rpm (6,200 × *g*) at 4°C.

   Pause Point: At this point the pellet can be flash-frozen and stored at −80°C.

13. Resuspend the pellets in a total of 100 mL ice-cold 2× PBS with 1 mM DTT, 20 μL benzonase (25 U/μL) and 1× protease inhibitors (2 x cOmplete™ EDTA-free Protease Inhibitor Cocktail tablets per 100 mL).
14. Lyse cells on ice using a high-pressure homogenizer (e.g., Emulsiflex) by passing the cell suspension through the homogenizer 2× at 1,500 bar.
15. Add NP-40 to a final concentration of 1%.
16. Clarify lysate by a centrifugation step in a JLA 16.250 rotor for 30 min at 16,000 rpm (38,000 × *g*) at 4°C.
17. Further clarify the supernatant by filtering through a 0.45 μm filter on ice.

   Note: From now on work in the cold room.

18. Equilibrate a Glutathione Sepharose High GSTrap HP column (total column volume is 50 mL) with 100 mL 2× PBS with 1 mM DTT at 5 mL/min.
19. Apply lysate to the GSTrap at 2 mL/min.
20. Wash with 10 column volumes (CV) 2× PBS, 1 mM DTT, 0.1% Tween20 at 10 mL/min.
21. Wash with 2 CV 5 mM ATP with 10 mM $MgCl_2$ in 2× PBS at 10 mL/min.
22. Incubate for 20 min.
23. Wash again with 2 CV 5 mM ATP with 10 mM $MgCl_2$ in 2× PBS at 10 mL/min.
24. Wash with 5 CV of 6× PBS and 1 mM DTT at 10 mL/min.
25. Wash with 5 CV of 2× PBS and 1 mM DTT at 10 mL/min.
26. Elute the GST-fusion protein using 5 mM reduced glutathione in 2× PBS and 1 mM DTT at pH 8.0 at 10 mL/min into ~12 fractions of 10 mL each.
27. Determine presence of protein by Bradford assay in the elution fractions.
28. Pool peak fractions to a maximum of 100 mL.
29. Dialyze against three changes of 100 mM $NaHCO_3$ with 100 mM NaCl at pH 8.2 (coupling buffer).

   Note: We do three rounds of dialysis (2 h, overnight, 2 h) with 2 L of coupling buffer each round.

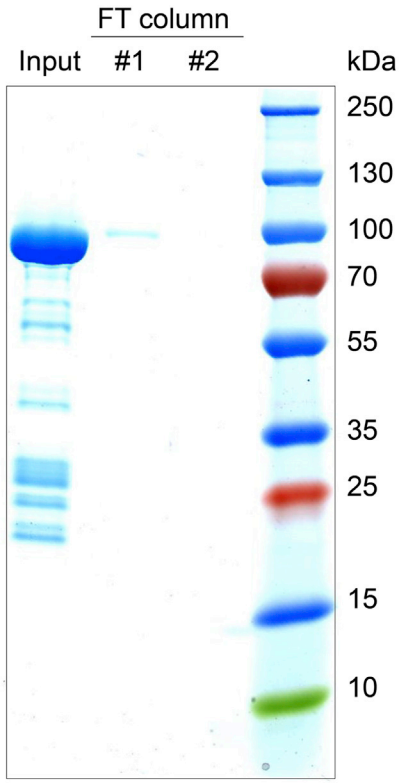

Figure 1. Purification and Coupling Efficiency of GST-TOG1/2

Expressed and purified GST-TOG1/2 (Input) used to make two TOG columns, flowthrough (FT) after conjugation to a NHS-column one (#1) and two (#2).

30. Equilibrate a 15 mL Amicon Ultra-4 concentration column with coupling buffer by spinning it 2× for 3 min at 4,000 rpm (3,220 × *g*) in a clinical centrifuge at 2°C.
31. Concentrate protein to a final volume of around 5 mL in a clinical centrifuge at 4,000 rpm (3,220 × *g*) at 2°C. The final concentration should be around 10 mg/mL.

Note: The expected molecular weight of the purified GST-TOG1/2 is ~94 kDa (Figure 1).

Conjugation of GST-TOG1/2 to an NHS Column

Note: We use a "HiTrap™ NHS-activated HP"-column with a total column volume of 5 mL and do the conjugation in a cold room.

32. Activate column with 2 CV freshly prepared ice-cold 1 mM HCl at 1 CV/min.
33. Add $MgCl_2$ to your concentrated GST-TOG1/2 protein solution, final concentration is 80 mM.
34. Load protein onto the NHS column and incubate for 20–30 min.
35. Wash with 6 CV 0.5 M Ethanolamine and 0.5 M NaCl at pH 8.3 at 1 CV/min to block remaining reactive groups.
36. Incubate for 30 min.
37. Wash with 10 CV 6× PBS at 1 CV/min.

Note: To determine how much of the GST-TOG1/2 bound to the column, we recommend taking samples before and after loading the protein on the column (Figure 1).

38. Wash with 5 CV 1× PBS with 50% glycerol (storage buffer) at 0.5 CV/min.
39. Store column at −20°C in a 50 mL Falcon tube.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-Tubulin (clone DM1A)	Sigma-Aldrich	Cat #T9026; RRID: AB_477593
Mouse monoclonal anti-Tubulin (clone B-5-1-2)	Sigma-Aldrich	Cat #: T5168; RRID: AB_477579
Tubulin C terminus with tyrosine removed	Abcam	Cat #: ab48389; RRID: AB_869990
Tyrosinated C terminus of tubulin	Abcam	Cat #: ab6160; RRID: AB_305328
Acetylated lysine 40 of α-tubulin (clone 6-11B-1)	Sigma-Aldrich	Cat #: T7451; RRID: AB_609894
Phosphorylated serine residues	Abcam	Cat #: ab9332; RRID: AB_307184
Bacterial Strains		
BL21(DE3) T1 pRARE	Sigma-Aldrich	Cat#: B2935
Chemicals, Peptides, and Recombinant Proteins		
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Merck	Cat #: 4693132001
Pregnant mare serum gonadotrophin (PMSG)	MSD, Tiergesundheit	Intergonan ® 240 IU/mL
Human chorionic gonadotrophin (HCG)	Sigma-Aldrich	Cat #: CG-10
Cytochalasin D	Sigma-Aldrich	Cat #: C8273
Xenopus laevis egg tubulin	Hirst et al., 2020	N/A
Xenopus tropicalis egg tubulin	Hirst et al., 2020	N/A
Xenopus borealis egg tubulin	This paper	N/A
Pluronic F-127	Sigma-Aldrich	Cat #: P2443
Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES)	Sigma-Aldrich	Cat #: P1851
NeutrAvidin Protein	Thermo-Fisher	Cat #: 31000
k-Casein from bovine milk	Sigma-Aldrich	Cat #: C0406
Chlorotrimethylsilane (TMCS)	Sigma-Aldrich	Cat #: 386529
GMPCPP	Jena Bioscience	Cat #: NU-405L
Protocatechuic Acid (PCA)	Sigma-Aldrich	Cat #: 03930590
Protocatechuate-3,4-dioxygenase (PCD)	Sigma-Aldrich	Cat #: P8279
Trolox	Sigma-Aldrich	Cat #: 238813
Cy3 Mono NHS Ester	GE Healthcare	Cat #: PA13101
Cy5 Mono NHS Ester	GE Healthcare	Cat #: PA15101
GTP	Sigma-Aldrich	Cat #: G8877
ATP	Sigma-Aldrich	Cat #: A26209

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
EGTA	Sigma-Aldrich	Cat #: E3889
EDTA	Carl Roth	Cat #: 8043.1
Sodium hydroxide (NaOH)	Carl Roth	Cat #: 9356.1
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Carl Roth	Cat #: HN04.3
Leupeptin	Sigma-Aldrich	Cat #: L2884
Pepstatin A	Sigma-Aldrich	Cat #: P5318
Chymostatin	Sigma-Aldrich	Cat #: C7268
Sodium chloride (NaCl)	Carl Roth	Cat #: HN00.3
Potassium chloride (KCl)	Carl Roth	Cat #: HN02.3
HEPES	Carl Roth	Cat #: HN77.5
Sucrose	Carl Roth	Cat #: 8890.4
Potassium hydroxide (KOH)	Carl Roth	Cat #: 6751.1
L-Cysteine	Carl Roth	Cat #: 1693.3
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	Carl Roth	Cat #: HN03.2
Gelatin from porcine skin	Sigma-Aldrich	Cat #: G1890
Glycerol	Carl Roth	Cat #: 3783.4
Ammonium sulfate ($(NH_4)_2SO_4$)	Carl Roth	Cat #: 3746.1
Bradford reagent	Sigma-Aldrich	Cat #: B6916
Benzonase	Merck Millipore	Cat #: 70664
DTT	Sigma-Aldrich	Cat #: 43815
Ampicillin	Carl Roth	Cat #: HP62.1
Chloramphenicol	Carl Roth	Cat #: 3886.2
Isopropyl-β-D-1-thiogalactopyranoside (IPTG)	Carl Roth	Cat #: 2316.3
Tween-20	Sigma-Aldrich	Cat #: P1379
Reduced glutathione	Sigma-Aldrich	Cat #: G4251
Sodium bicarbonate ($NaHCO_3$)	Carl Roth	Cat #: HN01.1
Hydrochloric acid (HCl)	Carl Roth	Cat #: 4625.2
Ethanolamine	Sigma-Aldrich	Cat #: E9508
NP40	Sigma-Aldrich	Cat #: 74385
Critical Commercial Columns and Consumables		
TOG1/2-column	Widlund et al., 2012	N/A
Glutathione Sepharose High Performance	GE Healthcare	Cat #: 11555035
HiTrap™ NHS-activated HP column (5 mL)	GE Healthcare	Cat #: GE17-0717-01
PD-10 desalting columns	GE Healthcare	Cat #: 17085101
Amicon Ultra-4 Centrifugal filter unit, 30 kDa (0.5 mL)	Merck Millipore	Cat #: UFC5030
Amicon Ultra-4 Centrifugal filter unit, 30 kDa (4 mL)	Merck Millipore	Cat #: UFC803024
Amicon Ultra-4 Centrifugal filter unit, 30 kDa (15 mL)	Merck Millipore	Cat #: UFC9030
Bottle top filtration unit, 0.22 µm	Thermo-Fisher	Cat #: 5954520
Syringe filtration unit, 0.45 µm	Carl Roth	Cat #: KC72.1

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Xenopus laevis	Nasco	Cat #: LM00535
Xenopus tropicalis	Nasco	Cat #: LM00823
Xenopus borealis	Nasco	Cat #: LM00699
Other - Equipment		
Ultra-clear centrifuge tubes for extract preparation	Beckman Coulter	Cat #: 344057
Rubber tube adapter for extract preparation	Beckman Coulter	Cat #: 870329
Polypropylene tubes for extract preparation	Beckman Coulter	Cat #: 331374
Polycarbonate ultracentrifuge tubes to clear extract	Beckmann Coulter	Cat #: 355647
1 mL syringe	Carl Roth	Cat #: H999.1
3 mL syringe	Carl Roth	Cat #: EP95.1
18G needle	Braun	Cat #: 4665120
27G needle	Braun	Cat #: 9186182
Peristaltic pump (REGLO Digital MS-4/8)	Ismatec	Cat #: ISM834C
Peristaltic pump tubing	Gilson	Cat #: F117942
Tubing connector to connect peristaltic pump tubing to the TOG column	Fisher Scientific	Cat #: 11300082
Recombinant DNA		
pGEX-6P-1 Stu2 1-590	Widlund et al., 2012	Addgene Plasmid #38314

MATERIALS AND EQUIPMENT

Preparing Materials/Equipment for Frog Injections and Egg Extract Preparation

For preparing egg extract you will need:

- Syringes (1 mL) and 27G-needles for frog injections
- Stock solution of hormones: PMSG (200 U/mL) and HCG (2,000 U/mL and 200 U/mL)
- Containers with lids to isolate frogs for egg laying
- Incubator to keep frogs at the appropriate temperature and in the dark while egg laying (optional)
- Glass beakers (0.8 or 1 L) for egg collection
- Plastic Pasteur pipette to sort and transfer eggs
- Ultracentrifuge tubes (5 mL, Beckman Coulter Cat #: 344057)
- Forceps
- Polypropylene centrifuge tube (13 mL, Beckman Coulter Cat #: 331374)
- Clinical centrifuge (for example Eppendorf 5810R with S-4-104 rotor) for packing spin
- High-speed centrifuge (e.g., Avanti JXN-26) with swinging bucket rotor (e.g., Beckman JS-13.1) for crushing spin
- Rubber adapters for high-speed rotor
- Syringe (1 or 2 mL) with 18G-needle for extract collection
- Eppendorf tubes
- Ice bucket with ice
- Box with cut pipette tips to handle the extract

Stock Solutions for Extract Preparation

Note: An overview of solutions for extract preparation is given in Table 2.

Table 2. *Xenopus* Egg Extract Buffers

Buffer	
10× MMR	Marc's Modified Ringer's, HEPES buffer at pH 7.8 to collect and wash eggs
20× XB-Salts	Stock solution of salts needed to prepare CSF-XB
CSF-XB Buffer	Cytostatic Factor Extract Buffer with XB-salts, extract buffer
CSG-XB+	CSF-XB Buffer with protease inhibitors
Dejelly Buffer	L-Cysteine solution to remove jelly coat from eggs

0.5 M EGTA/0.5 M EDTA

Prepare a stock solution by combining

- 19.02 g of EGTA, or
- 18.61 g of EDTA
- 90 mL of ultra-pure water

Mix with the help of a stir bar. To dissolve either solution, slowly add NaOH to a pH of 8.0. Once the salt has dissolved adjust the final volume to 100 mL using ultra-pure water. Filter-sterilize or autoclave both solutions and store at room temperature.

1 M $CaCl_2$

Prepare stock solution by combining

- 14.7 g of $CaCl_2.2H_2O$
- 90 mL of ultra-pure water

Stir and dissolve with the help of a stir bar. Adjust the final volume to 100 mL using ultra-pure water. Autoclave and store at room temperature.

1 M $MgCl_2$

Prepare stock solution by combining

- 20.33 g of $MgCl_2.6H_2O$
- 90 mL of ultra-pure water

Stir and dissolve with the help of a stir bar. Adjust the final volume to 100 mL using ultra-pure water. Autoclave and store at room temperature.

Inhibitor Stocks for Extract Preparation

- Dissolve Cytochalasin D (10 mg/mL) in DMSO. Prepare 25 µL aliquots and store at −20°C.

  Note: Cytochalasin D is added to cytostatic factor (CSF) extracts to prevent actin polymerization. Actin-intact CSF extracts undergo gelation when incubated at room temperature (Field et al., 2011), which makes them more viscous and harder to work with. Methods for preparing actin-intact egg extracts are described in (Field et al., 2014, Field et al., 2017).

- Dissolve 1 tablet of cOmplete™, EDTA-free Protease Inhibitor Cocktail in 0.5 mL of CSF-XB Buffer. Store aliquots at −20°C for up to 3 months.

Alternatives: Instead of the protease inhibitor tablets, one can prepare an LPC cocktail. Dissolve

- 10 mg/mL leupeptin
- 10 mg/mL pepstatin A
- 10 mg/mL chymostatin
- in DMSO

Prepare 100 μL aliquots and store at −20°C.

10× MMR Buffer

10× MMR Buffer contains 1 M NaCl, 20 mM KCl, 10 mM $MgCl_2$, 20 mM $CaCl_2$, 1 mM EDTA, and 50 mM HEPES, at pH 7.8. To prepare a 5 L stock combine:

- 292.2 g of NaCl
- 7.46 g of KCl
- 10.17 g of $MgCl_2$
- 11 g $CaCl_2$
- 10 mL of the 0.5 M EDTA stock solution
- 59.58 g of HEPES
- 4.8 L of ultra-pure water

Mix all components with the help of a stir bar. Adjust pH to 7.8 with NaOH. Adjust the final volume to 5 L using ultra-pure water. Store at room temperature for several months.

Note: When preparing a 1× or 0.5× MMR from the 10× stock solution ensure that the pH of the working solution is still 7.8. Adjust pH with NaOH if required.

20× XB-Salt Solution

20× XB- salt solution contains 2 M KCl, 20 mM $MgCl_2$, and 2 mM $CaCl_2$. To prepare a 1 L stock combine:

- 149.12 g of KCl
- 20 mL of the 1 M $MgCl_2$ stock solution
- 2 mL of the 1 M $CaCl_2$ stock solution
- 0.9 L of ultra-pure water

Mix components with the help of a stir bar. Adjust the final volume to 1 L using ultra-pure water. Filter-sterilize the solution and store at 4°C for several months.

CSF-XB Buffer

We recommend preparing 0.5 L for each batch of laid eggs freshly before the extract prep.

CSF-XB Buffer contains 10 mM HEPES, 50 mM Sucrose, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ (present in XB-salt solution) and 5 mM EGTA, at pH 7.7. To prepare 1 L of CSF-XB combine:

- 2.38 g of HEPES
- 17.11 g of Sucrose
- 50 mL of the 20× XB-salt solution
- 10 mL of the 0.5 M EGTA stock
- 0.9 L of ultra-pure water

Mix components with the help of a stir bar. Adjust the pH to 7.7 with KOH and adjust the final volume to 1 L using ultra-pure water. Store the buffer between 16°C–18°C in an incubator.

CSF-XB+ Buffer

We suggest preparing 50 mL of CSF-XB+ Buffer per batch of eggs by adding protease inhibitors to CSF-XB Buffer:

- 100 mL of the above CSF-XB Buffer
- 1 mL of the Protease Inhibitor Cocktail solution.

Alternatives: When using the LPC cocktail. Combine:

- 100 mL of CSF-XB Buffer
- 100 µL of LPC cocktail

Mix thoroughly.

Dejelly Buffer

We recommend preparing 0.5 L per batch of laid eggs freshly before (maximum 1 h) the extract prep.

Note: The Dejelly Buffer is different for each frog species. *X.l.* eggs are dejellied using 2% (w/v) Cysteine in 0.25× MMR, *X.b.* eggs using 3% Cysteine in 0.25× MMR and *X.t.* using 3% Cysteine with no salt.

- 20 g of L-Cysteine for a 2% solution (*X.l.*), or 30 g of L-Cysteine for a 3% solution (*X.b.*, *X.t.*)
- 25 mL of 10× MMR Buffer (*X.l.*, *X.b.*) or water (*X.t.*)
- 0.9 L of ultra-pure water

Mix components with the help of a stir bar. Adjust the pH to 7.8 with NaOH and adjust the final volume to 1 L using ultra-pure water. Store the buffer between 16°C–18°C in an incubator.

Preparing Materials/Equipment for Affinity Purification of Tubulin

To clear the egg extract before loading it onto the TOG column, you will need:

- High-speed centrifuge (e.g., Beckman Coulter Optima Max-XP) with a fixed-angle rotor (e.g., Beckman MLA-80)
- Polycarbonate ultracentrifuge tubes (6.5 mL, Beckman Coulter Cat #: 355647)

To assemble the purification setup in the cold room, you will need;

- Peristaltic pump (e.g., REGLO Digital MS-4/8)
- Peristaltic pump tubing
- Tubing connector to connect tubing to the TOG column
- Glass beaker to collect liquid waste
- PD-10 desalting columns
- Pipette boy
- Serological pipettes (10 mL)
- Labeled Eppendorf tubes for taking SDS-PAGE and western blot samples and for collecting fractions
- Laemmli sample buffer for SDS-PAGE and western blot samples
- Set of pipettes and filter-tips
- Tube racks

Stock Solutions and Buffers for Tubulin Purification

Note: An overview of stock solutions and buffers for tubulin purification is given in Table 3.

Table 3. Tubulin Purification Buffers

Buffer	
1× BRB80	The only buffer tubulin is happy in
Wash buffer	1× BRB80 with GTP to reduce binding of unspecific proteins
ATP Wash Buffer	1× BRB80 with ATP to remove chaperones
Elution Buffer	High-salt buffer to elute tubulin off the TOG domains
Desalting Buffer	1× BRB80 with GTP, also tubulin storage buffer
Column Storage Buffer	Glycerol in PBS allows the TOG column to be stored at −20°C

1 M DTT

- 1.54 g DTT
- 10 mL ultra-pure water

Store 100 µL aliquots at −20°C.

1 M $MgCl_2$

Prepare stock solution by combining

- 10.15 g of $MgCl_2 \cdot 6H_2O$
- 45 mL of ultra-pure water

Stir and dissolve with the help of a stir bar. Adjust the final volume to 50 mL using ultra-pure water. Filter through a 0.45 µm filter and store at 4°C.

0.5 M EGTA

Prepare a stock solution by combining

- 3.8 g of EGTA
- 15 mL of ultra-pure water

Mix with the help of a stir bar. To dissolve the solution, slowly add NaOH to a pH of 8.0. Once the salt has dissolved adjust the final volume to 20 mL using ultra-pure water. Filter-sterilize through a 0.45 µm filter and store at 4°C.

100 mM GTP

- 0.52 g Guanosine 5′-triphosphate (GTP) sodium salt hydrate
- 7 mL ultra-pure water

Mix at room temperature and adjust to pH 7.0 with NaOH. Use 1 M NaOH until the pH reaches 6.5, then add 0.1 M NaOH slowly until the pH reaches 7.0. Add ultra-pure water to a final volume of 10 mL, distribute into single-use aliquots (500 µL) and freeze at −20°C.

100 mM Mg^{2+}ATP

- 0.55 g Adenosine 5′-triphosphate (ATP) disodium salt hydrate
- 8.5 mL ultra-pure water

- 1 mL 1 M $MgSO_4$

Adjust to pH 7.0 with NaOH. Use 1 M NaOH until the pH reaches 6.5, then add 0.1 M NaOH slowly until the pH reaches 7.0. Add ultra-pure water to a final volume of 10 mL, aliquot into single-use volumes of 1 mL and freeze at −20°C.

> ***Note:*** These nucleotides undergo rapid hydrolysis at acidic pH, therefore monitor pH using a small-volume pH probe while dissolving and keep pH close to neutral. The GTP solution should be made without Mg^{2+}, which will cause precipitation.

1× BRB80

1× BRB80 buffer contains 80 mM PIPES, 1 mM $MgCl_2$, and 1 mM EGTA, pH 6.9. To prepare 500 mL of 1× BRB80 buffer, combine:

- 12.1 g of PIPES
- 1 mL of 0.5 M EGTA
- 500 µL of 1 M $MgCl_2$
- 8 mL of 10 M KOH
- 450 mL of ultra-pure water

Stir the PIPES, adjust the pH to 6.9 using 10 M KOH and then adjust the final volume to 500 mL with ultra-pure water. PIPES will only dissolve while adjusting pH. Next, filter through a 0.22 µm filter and store the buffer at 4°C. We prepare 1× BRB80 the day before the tubulin purification.

> ***Optional:*** Alternatively, you can prepare a 5× stock of BRB80 and keep it at −20°C for longer periods of time until the day of the tubulin purification. After thawing, make sure the pH is 6.9.

> ⚠ CRITICAL: The following buffers are prepared shortly before starting the tubulin purification and kept on ice at all times. ATP and GTP are kept on ice and added to the buffers shortly before use.

Wash Buffer

Wash buffer contains 1× BRB80 and 100 µM GTP.

- 200 mL 1× BRB80
- 200 µL 100 mM GTP

Mix by inverting.

ATP Wash Buffer

ATP wash buffer contains 1× BRB80, 100 µM GTP, 10 mM $MgCl_2$ and 5 mM Mg^{2+}ATP.

- 37.56 mL 1× BRB80
- 40 µL 100 mM GTP
- 400 µL 1 M $MgCl_2$
- 2 mL 100 mM Mg^{2+}ATP

Mix by inverting.

Elution Buffer

Elution buffer contains 1× BRB80, 100 µM GTP and 500 mM Ammonium sulfate.

- 30 mL 1× BRB80

- 30 μL 100 mM GTP
- 2 g Ammonium sulfate

Mix by inverting and make sure that the Ammonium sulfate is completely dissolved. Make sure the pH is 6.9.

Desalting Buffer

This is the final tubulin storage buffer, which contains 1× BRB80 and 10 μM GTP.

- 100 mL 1× BRB80
- 10 μL 100 mM GTP

Mix by inverting.

Column Storage Buffer

- 25 mL 1× PBS
- 25 mL glycerol

Mix by inverting and make sure there are no air bubbles in the buffer before using it on the TOG column.

Experimental Model and Subject Details

The *Xenopus* frogs (adult females) used in this study are part of the *Xenopus* colony maintained at the animal husbandry of the Humboldt-Universität zu Berlin. Mature *X. laevis*, *X. tropicalis* and *X. borealis* frogs were obtained from NASCO (Fort Atkinson, WI). *Xenopus* frogs were maintained in a recirculating tank system with regularly monitored temperature and water quality (pH, conductivity, and nitrate/nitrite levels). *X. laevis* were housed at a temperature of 18°C–20°C, *X. tropicalis* were housed at 23°C–26°C, and *X. borealis* were housed at 21°C–23°C. Frogs were fed with food pellets (V7106-0202) from ssniff Spezialdiäten GmbH. All experimental protocols involving frogs were performed in accordance with national regulatory standards and ethical rules and reviewed and approved by the LaGeSo under Reg.-Nr. 0096/15.

STEP-BY-STEP METHOD DETAILS

Figure 2 shows an overall schematic of the workflow.

Preparing *Xenopus* Egg Extracts

Timing: 2 h

Prepare egg extracts from different frog species (*X.l.*, *X.b.*, and *X.t.*).

Extract preparation is a time-sensitive process requiring constant vigilance. An experienced researcher can handle 5–6 batches of eggs at the same time. For scaling up, we suggest involving multiple lab members. We recommend preparing extracts in a temperature-controlled lab-space maintained between 18°C–20°C.

⚠ CRITICAL: For *X.t.* extract preparation, all glass and plastic-ware must be coated by swirling a 0.2% gelatin % (w/v) solution in the beakers. Discard gelatin solution from beakers after use.

1. Collect laid eggs in a 0.8 or 1 L glass beaker (Figure 3A) and keep batches separate if possible.

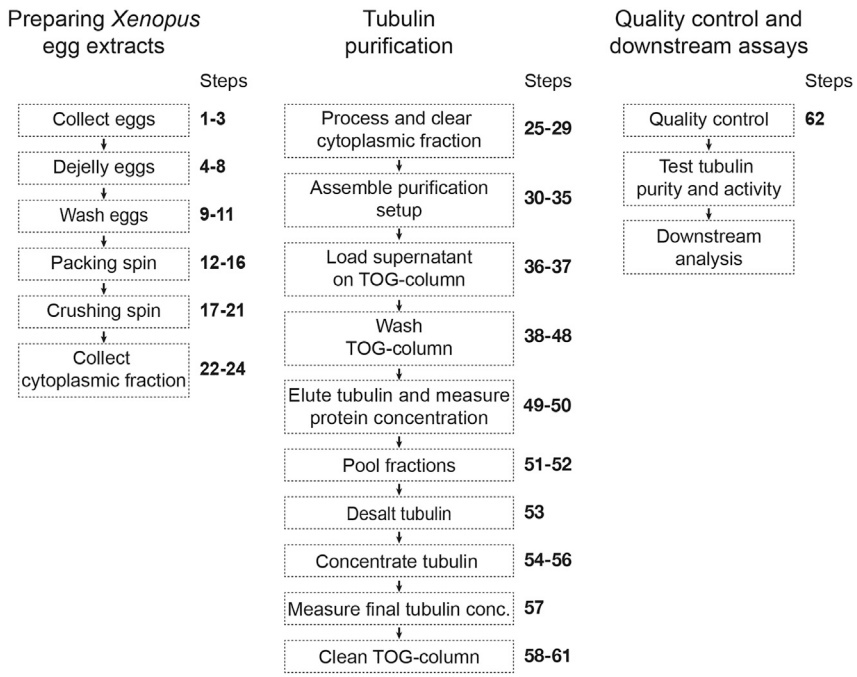

Figure 2. Overall Schematic Workflow

For each stage (dotted box), steps are annotated.

Note: For *X.l.*, the frogs are kept in 1× MMR during egg laying. *X.b.* frogs on the other hand are kept in 0.5× MMR. *X.t.* frogs are kept in tank water, but eggs are collected in 1× MMR. Both laid and squeezed eggs from each species can be used for extract preparation.

2. Pour off any residual buffer/water and wash the eggs several times in 1× MMR (0.5× MMR for *X.b.*).
3. With the help of a Pasteur pipette remove lysed and puffy eggs (Figure 3B).

⚠ CRITICAL: The quality of eggs is important and can impact the yield and quality of protein obtained. Sorting out puffy and lysed eggs (Figure 3B) should thus be performed meticulously. Additionally, we suggest discarding any batch of eggs with more than 10% of total eggs lysed or activated.

4. Pour off the MMR and add Dejelly Buffer.
5. Gently swirl the beaker to ensure uniform dejellying. As the dejellying successfully progresses, the eggs become more densely packed.
6. Exchange the solution after 2.5 min and wash again with fresh Dejelly Buffer.
7. Upon successful dejellying, the eggs pack tightly (Figure 3D).

⚠ CRITICAL: Ensure that the dejellying step is no longer than 5–6 min (10 min for *X.t.* eggs). Excessive dejellying may damage the eggs and cause lysis.

8. Pour off as much Dejelly Buffer as possible while ensuring that the eggs are not exposed to air.
9. Add CSF-XB to the beaker and gently swirl the eggs. CSF-XB addition halts the dejellying reaction.

⚠ CRITICAL: Dejellied eggs are particularly sensitive to mechanical perturbations. Special care must thus be taken during the following steps.

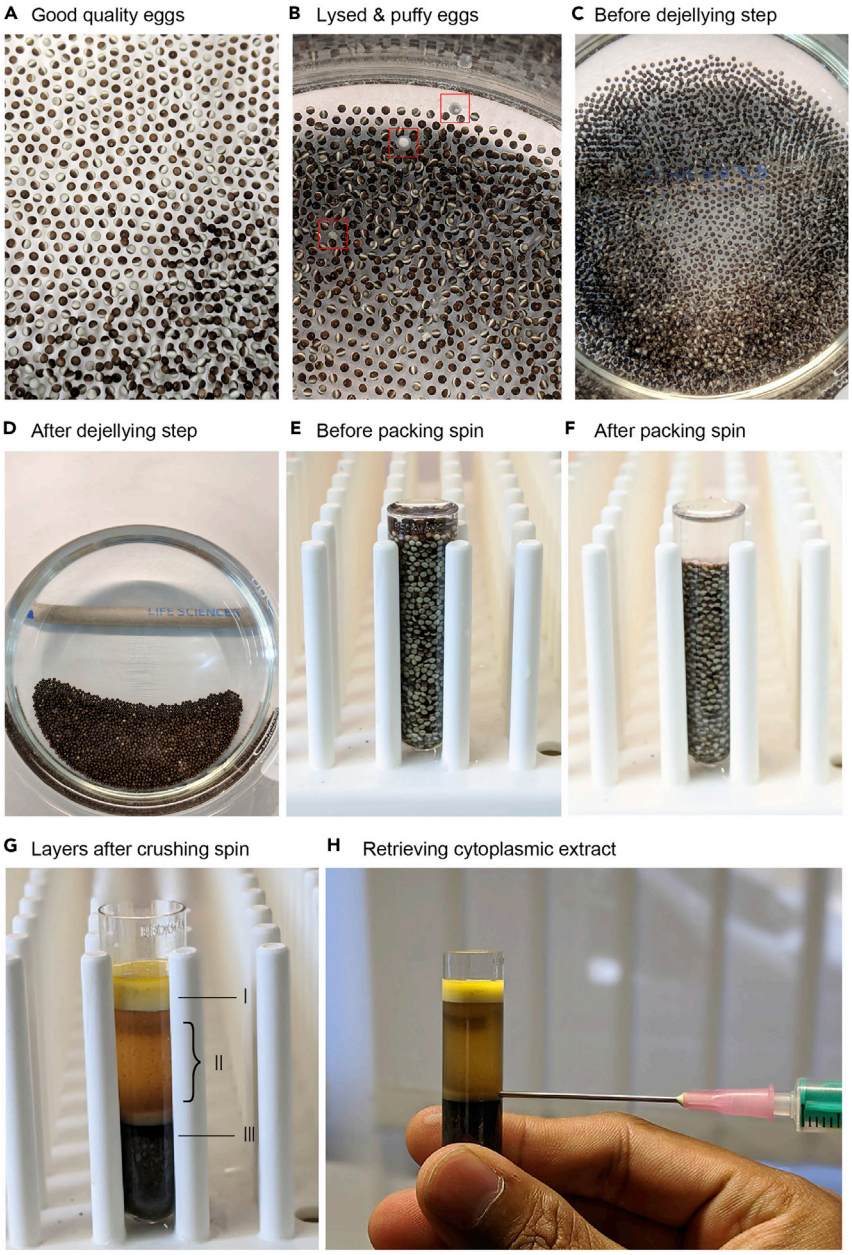

Figure 3. Preparing *Xenopus* Egg Extract

(A) Good-quality *Xenopus laevis* eggs.

(B) Lysed and puffy eggs (in red squares).

(C) Sorted eggs that have been washed with MMR Buffer.

(D) After successful dejellying, eggs are densely packed.

(E) Eggs transferred into a suitable centrifuge tube.

(F) After a short low-speed spin eggs are tightly packed.

(G) A subsequent high-speed spin is used to crush the eggs and fractionate them into different layers: layer I is enriched in lipids, layer II is critical as it contains all cytoplasmic proteins including tubulin and layer III contains a mixture of pigment granules, yolk, nuclei, and egg cortex.

(H) With the help of a syringe and 18G needle, layer II is extracted.

10. Decant the CSF-XB and wash 2–3 times with CSF-XB Buffer.
11. Exchange the CSF-XB for CSF-XB+ buffer (CSF-XB containing protease inhibitor) and gently swirl.
12. Preload an ultracentrifuge tube with 1 mL of CSF-XB+ buffer and 10 μL of Cytochalasin D (10 mg/mL) (*X.l.* and *X.b.*). For *X.t.* eggs add 1 mL of CSF-XB+ and 5 μL of Cytochalasin D.
13. Carefully load eggs into the tube with the help of a cut plastic Pasteur pipette.

Note: Ensure that while transferring eggs into the tube they are not exposed to air and try to fill the tube all the way to the top (Figure 3E). If eggs from different batches appear healthy up to this step, they can be mixed to ensure centrifuge tubes are filled completely. Full tubes provide better separation during the crushing spin.

14. Transfer the ultracentrifuge tube into a polypropylene tube with the help of forceps. The polypropylene tube acts as an adaptor that fits most clinical centrifuges (Eppendorf, Sorvall, etc.).

Note: Place a few drops of buffer into the polypropylene adaptor before transferring the tube containing eggs to ensure the tube does not stick to the adaptor after the spin.

15. Packing spin: Transfer tubes to clinical centrifuge set to 18°C (*X.l., X.b.*) or 16°C (*X.t.*). Spin *X.l.* and *X.b.* eggs at 500 rpm for 30 s, followed by 2,000 rpm for 90 s. *X.t.* eggs are spun at 200 rpm for 30 s, followed by 800 rpm for 90 s.
16. After this spin, eggs should appear tightly packed (Figure 3F).
17. Remove any residual buffer on top of the eggs.

⚠ CRITICAL: It is important to remove any residual buffer from the top of the eggs. At this step it is okay to sacrifice some of the eggs by exposing them to air, as any residual buffer will dilute the extract.

18. Crushing spin: Transfer the tubes into rubber adaptors. Spin at 11,000 rpm in a JS-13.1 swinging bucket rotor (19,000 × *g*) for 20 min.

⚠ CRITICAL: Centrifugation should start at room temperature or 18°C. For *X.l.* egg extracts, the centrifuge should cool down during the spin to reach 4°C. For *X.b.* and *X.t.* egg extracts, the temperature can stay at constant 16°C–18°C. Extracts are also temperature sensitive and the correct temperature must be maintained to ensure proper biochemical activity. The acceleration settings for the centrifuge are set to maximum. The deceleration step is set to slow and the brake is turned off.

19. Remove the thin-wall tube with the fractionated eggs from the polypropylene adaptor.
20. After the crushing spin, the eggs should separate into three distinct layers (Figure 3G). The top layer is enriched in lipids. The second golden layer is the cytoplasmic layer and contains all soluble cytoplasmic proteins. The final dark layer contains a mixture of pigment granules, yolk, and egg cortex.

⚠ CRITICAL: All equipment used to handle extracts should be kept at the appropriate temperature. For *X.l.* and *X.b.* extracts, syringes, needles, forceps, tubes, etc. should be kept on ice. For *X.t.* extracts, equipment can be kept between 16°C–20°C.

21. With the help of a syringe and 18G-needle, puncture the tube near the bottom of the cytoplasmic layer (Figure 3H), rotate the needle so that the opening faces up and and gently aspirate the cytoplasmic fraction.
22. Remove the needle and gently expel the extract into an Eppendorf tube.

⚠ CRITICAL: For purification of tubulin, all extracts should be kept on ice. However, if you plan to assemble RanGTP-asters or spindles to test extract quality, you can keep aliquots of *X.l.* and *X.b.* egg extract on ice and *X.t.* egg extract at 16°C. Extract can be used for 6–8 h without significant loss of activity.

23. Add Cytochalasin D (1:1,000) and protease inhibitors (1:100) to the *X.l.* extract. To *X.b.* and *X.t.* extract, add Cytochalasin D (1:500) and protease inhibitors (1:200).
24. Gently mix the extract with the help of a cut pipette tip.

Optional: Take a western blot sample of your extract for quality control (see tubulin post-translational modifications).

⚠ CRITICAL: We recommend proceeding to the tubulin purification step immediately. Extract should not be frozen at this point, as this will impact tubulin activity.

TOG Affinity Purification of Tubulin from *Xenopus* Egg Extract

Timing: 6 h from clearing extracts to frozen aliquots of purified tubulin

Perform all following steps in a cold room or on ice. Pre-chill all buffers, containers, columns etc.

Note: As a reference we will use a total of 4 mL egg extract and a 5 mL TOG column. Yields for the various purifications are shown in Table 1.

25. Dilute egg extract 1:1 with 1× BRB80 to a final volume of 8 mL.
26. Take a 10 μL sample "lysate" for SDS-PAGE and western blot analysis.
27. Add 3 μL benzonase (25 U/μL) and DTT to 1 mM final concentration and mix carefully by slowly pipetting up and down with a cut pipette tip.
28. Split the diluted egg extract into two ice-cold polycarbonate ultracentrifuge tubes and adjust to equal weight by adding small drops of 1× BRB80.
29. Centrifuge at 80,000 rpm (440,000 × *g*) in a pre-cooled MLA-80 rotor at 2°C for 30 min.

Note: After the spin, the extract should be clear. If this is not the case, you can further clear the lysate by passing it through a 0.45 μm filter. In case a layer formed on top of the cleared extract, collect the lysate carefully with a syringe and a needle.

30. Take a 10 μL sample of "HS spin" for SDS-PAGE and western blot analysis.
31. During the spin, assemble the purification setup in the cold room.
32. Wash tubing with 20% ethanol.
33. Wash tubing with ultra-pure water.
34. Wash tubing with wash buffer.
35. Connect the TOG column to the peristaltic pump and tubing and equilibrate the TOG column with 10 CV 1× BRB80 at a flow rate of 1 CV/min.

⚠ CRITICAL: Be careful not to introduce air bubbles into the TOG column when connecting the tubing, otherwise you compromise TOG activity. If you happen to have air bubbles in the tubing, disconnect the tubing from TOG column and pump wash buffer through the tubing until the air bubbles are out of the system, then reconnect to the TOG column carefully. Never let the TOG column run dry.

36. Cycle the cleared lysate through the TOG column at a flow rate of 0.5 CV/min for 20 min.
37. Take a 10 μL sample of "flow-through" for SDS-PAGE and western blot analysis.
38. Wash with 2 CV wash buffer to clear out most of the extract from the TOG column.

39. Take a 100 µL sample of "wash 1" for SDS-PAGE and western blot analysis.
40. Change the flow rate to 1 CV/min and wash with 10 CV wash buffer.
41. Take a 100 µL sample of "wash 2" for SDS-PAGE and western blot analysis.
42. Wash with 3 CV ATP wash buffer.
43. Take a 100 µL sample of "ATP wash" for SDS-PAGE and western blot analysis.
44. Incubate for 15 min.
45. Wash with 3 CV ATP wash buffer.
    a. Meanwhile you can start equilibrating the PD-10 desalting columns 3× with ice-cold desalting buffer.

Note: Each PD-10 column can hold 2.5 mL of eluted tubulin and 3.5 mL desalting buffer are used to elute it from the column. We usually calculate to have a maximum of 10 mL of pooled tubulin fractions, so we calibrate a total of 4 PD-10 columns.

46. Take a 100 µL sample of "wash 4" for SDS-PAGE and western blot analysis.
47. Wash with 10 CV wash buffer.
48. Take a 100 µL sample of "wash 5" for SDS-PAGE and western blot analysis.

Note: At this step, there should not be any protein in your washes. You can check this wash for the presence of proteins either by Bradford assay or A_{280}. If you detect protein, additionally wash with 4 CV wash buffer. Take a 100 µL sample of "wash 6" for SDS-PAGE and western blot analysis.

49. Decrease flow rate to 0.25 CV/min and elute tubulin with 3 CV elution buffer into 15× 1 mL fractions.
50. Check each fraction for protein by using a Bradford assay or A_{280}.
51. Pool the fractions with the highest protein concentration.

⚠ CRITICAL: Take care to limit exposure of the tubulin to high salt since this will compromise tubulin activity over time. Quickly desalt the pooled fractions into desalting buffer using the PD-10 columns.

52. Take a 10 µL sample of "pooled peak elutions" for SDS-PAGE and western blot analysis.
53. Desalt pooled fractions via the PD-10 columns using ice-cold desalting buffer.
54. Equilibrate one 4 mL Amicon Ultra-4 concentration column with desalting buffer by spinning it 2× for 3 min at 4,000 rpm (3,220 × *g*) in a clinical centrifuge at 2°C.

⚠ CRITICAL: The concentration step is the most critical in our hands. In some cases, we have lost up to 30% protein during this step. To minimize loss, we recommend thoroughly equilibrating the concentration column with desalting buffer and avoiding long spinning periods (See also Troubleshooting).

55. Concentrate the tubulin to at least 20 µM using a 4 mL Amicon Ultra-4 concentration column by spinning in a clinical centrifuge at 4,000 rpm (3,220 × *g*) at 2°C.

Note: A 4 mL Amicon Ultra-4 concentration column allows you to concentrate to a minimum volume of ~500 µL. In case you want to concentrate further, we recommend switching to a smaller concentration column with a maximum capacity of 0.5 mL.

56. Take a 2 µL sample of "desalted and concentrated" for SDS-PAGE and western blot analysis.
57. Once tubulin is concentrated to your desired concentration, measure the final protein concentration, flash-freeze in liquid nitrogen and store at −80°C. We usually use PCR tubes and make 5 µL aliquots.

Note: Per convention, tubulin concentration is given for the αβ-dimer (ϵ_{280}= 115,000 M^{-1} cm^{-1} and MW= 110 kDa).

58. Clean the TOG column by washing with 5 CV 1× PBS at 1 CV/min.

Note: Ideally the TOG column is cleaned directly after use. We usually clean it during the concentration step.

59. Lower the flow rate to 0.5 CV/min and wash with 10 CV 10× PBS.
60. Wash with 10 CV column storage buffer.
61. Cap column tightly without introducing air bubbles and store in a 50 mL Falcon tube at −20°C.
62. Proceed with SDS-PAGE and western blot analysis for quality control.

EXPECTED OUTCOMES

This protocol allows for the purification of pure and highly concentrated active tubulin. To document and ensure the quality of each purification step, we usually take a number of quality control steps: (1) SDS-PAGE and Coomassie stain of the tubulin purification process, (2) SDS-PAGE and western blot analysis against α-tubulin of the tubulin purification process, (3) SDS-PAGE and western blot analysis against different post-translational modifications of extract and purified tubulin to show the tubulin's post-translational modification pattern was conserved throughout the purification, (4) in-solution digest and mass spectrometry to assess tubulin purity, (5) polymerization assay to test tubulin activity, (6) reconstitution of microtubule dynamics *in vitro* by TIRF.

SDS-PAGE and Western Blot Analysis of α-Tubulin

To determine the effectiveness of the affinity purification, resolve the samples taken during the purification via SDS-PAGE and stain the gel with Coomassie (Figure 4A). Load sample volumes corresponding to 1 μL extract from each step in order to determine tubulin loss during the spin, in the flow-through and washes, and to ascertain final sample purity, and tubulin enrichment relative to the input:

1. Lysate
2. HS spin
3. Flow-through
4. 1st wash
5. 2nd wash
6. ATP wash
7. 4th wash
8. 5th wash
9. 6th wash
10. Pooled peak elutions
11. Desalted and concentrated

In the Coomassie, lanes 5–9 should not contain substantial amounts of protein. Lanes 10 and 11 should not contain any other protein than tubulin, which migrates as a closely spaced doublet at 55 kDa. Bands that run lower than 55 kDa are most likely tubulin degradation products, which will also appear in the western blot analysis.

In a second step, we analyse the same samples by western blot (Figure 4B) using a pan-specific anti–α-tubulin monoclonal antibody (DM1α). Here, lanes 1 and 2 should contain comparable amounts of tubulin, indicating no substantial tubulin loss during the high-speed spin. Importantly, lane 3 should not contain any tubulin, indicating a complete depletion of tubulin from the lysate and thus no enrichment of a tubulin subpopulation, e.g., a specific isoform or post-translational modification.

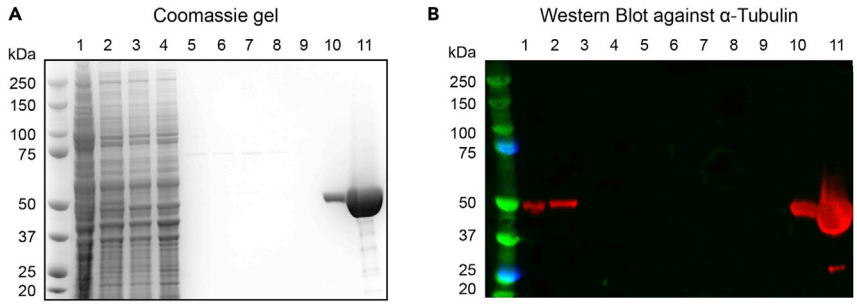

Figure 4. Coomassie Staining and Western Blot Analysis of the Tubulin Purification

(A) Coomassie-stained SDS-PAGE of samples taken during the purification of tubulin. (1) Lysate; (2) HS spin; (3) Flow-through; (4) 1st wash; (5) 2nd wash; (6) ATP wash; (7) 4th wash; (8) 5th wash; (9) 6th wash; (10) Pooled elutions; (11) Desalted and concentrated tubulin.

(B) Western blot with antibody against α-tubulin of the same samples as in (A).

Tubulin Post-translational Modifications

To make sure that the purification did not change the tubulin's post-translational modification pattern, we probe extract tubulin (sample taken at step 26) and purified tubulin (sample taken at step 56) by western blot using antibodies against α-tubulin as loading control, tyrosinated tubulin (Tyr), detyrosinated tubulin (Detyr), acetylated lysine 40 (K40), and phosphoserine (P-Ser) (Figure 5A).

Tubulin In-Solution Digest and Mass Spectrometry

Tubulin sample purity can be evaluated by LC-MS/MS analysis of in-solution tryptic digests of the purified tubulin. We use the emPAI (Exponentially Modified Protein Abundance Index) value to estimate the relative abundance of the proteins in our sample. Tubulin usually is of high purity (> 98%). If additional proteins are identified, they mainly include highly abundant proteins like heat shock proteins or ribosomal proteins. There should not be any contamination with microtubule associated proteins (MAPs) or motor proteins, as these will influence microtubule dynamics in your downstream experiments.

Polymerization Assay

For any tubulin purification, it is essential to confirm that the purified protein is polymerization-competent. The simplest way to do this is a tubulin polymerization assay. Purified tubulin (e.g., 20 μM) is induced to polymerize in 1× BRB80 buffer in the presence of 1 mM GTP and 33% v/v glycerol at 35°C for 40 min. The reaction is layered on top of an equal volume of 1× BRB80 containing 60% glycerol, and centrifuged in a TLA-100 rotor at 35°C for 10 min at 80,000 rpm (280,000 × *g*). Polymerized microtubules form the pellet, whereas non-polymerized tubulin remains in the reaction mixture above the 60% glycerol layer. Relative tubulin abundance in the input, supernatant, and pellet fractions are assessed by SDS-PAGE and western blot analysis. Most of the total tubulin (>90%) should be found in the pelleted polymer fraction (Figure 5B).

> ***Note:*** Tubulin concentration and assay temperature for microtubule polymerization will dependent on the source of the purified tubulin. *X. laevis* tubulin will readily polymerize at 20°C and 10 μM, while conventional bovine brain tubulin will not.

This polymerization assay, however, cannot distinguish between tubulin assembled into microtubules or sheets. To determine whether the affinity-purified tubulin forms microtubules that undergo dynamic instability, we recommend reconstituting dynamic microtubules *in vitro* and visualizing them by TIRFM or IRM.

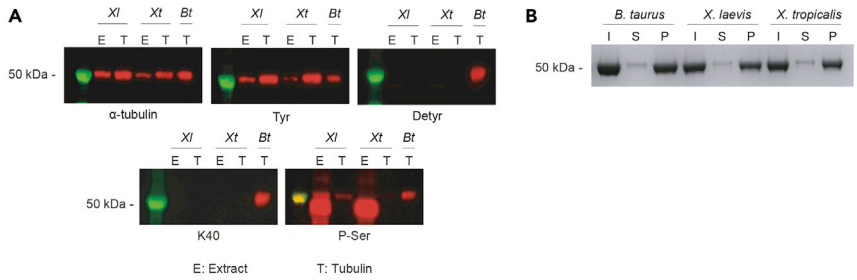

Figure 5. Posttranslational Modifications and Activity of Purified Tubulin

(A) Western blot against tubulin PTMs before and after the purification. 300 ng tubulin for each species (*Bos taurus, Xenopus laevis, Xenopus tropicalis*) were loaded onto a polyacrylamide gel adjacent to a corresponding volume of egg extract. Samples were transferred to a nitrocellulose membrane and probed with antibodies against α-tubulin, tyrosinated tubulin (Tyr), detyrosinated tubulin (Detyr), acetylated lysine 40 (K40), and phosphoserine (Pser).

(B) Tubulin polymerization assay for assessing the relative activities of bovine brain tubulin and *Xenopus* tubulin purified via the TOG column. 2 μL of the 20 μM initial reaction mixture was loaded onto the gel as the input (I), followed by equivalent volumes for the supernatant (S) and the pellet (P) after resuspending the pellet in the same starting volume of 1× BRB80.

LIMITATIONS

The final amount of tubulin will mainly depend on the following three factors:

(1) The total amount of tubulin in your starting material/lysate,
(2) tubulin binding to TOG column depending on its binding capacity,
(3) efficiency of tubulin elution, desalting, and concentration.

We usually start with a back-of-the-envelope calculation to estimate the tubulin present in our starting material. For *Xenopus* egg extracts, we recommend using a minimum of 2 mL of egg extract to purify enough tubulin for downstream functional assays. The binding capacity of the TOG column will depend on the number of immobilized TOG1/2 domains, and each TOG1/2 will bind one tubulin heterodimer (Brouhard et al., 2008, Widlund et al., 2011). Finally, the efficiency of tubulin binding, elution, desalting, and concentration can be monitored as described above and improved as described below.

TROUBLESHOOTING

Problem

Frogs have not laid enough eggs or eggs of poor quality.

Potential Solution

If the frogs have not laid enough eggs before the start of the experiment, we suggest waiting a few hours and delaying the entire purification process. In our experience, giving the frogs a gentle squeeze can help accelerate the egg-laying process. Additionally, frogs can lay a second batch of eggs several hours after the first batch has been laid. Priming the frogs maximizes the chances of ovulation, increases egg yield, and improves egg quality. Frogs should be given a 3-month recovery period between ovulations.

Problem

No distinct layers after crushing spin.

Potential Solution

For the crushing spin, make sure to set the acceleration to maximum, the deceleration to slow, and to turn the brake off. For *X.l.* eggs, starting with a rotor at room temperature and only cooling down during the spin improves layer separation.

Problem
Extract is too dilute.

Potential Solution
Ensure that most of the buffer is removed while exchanging solutions during the extract preparation. Also remove any buffer left on top of the eggs after the packing spin. Any residual buffer left will dilute the concentration of proteins in the extract. Final egg extract concentration should be 100 mg/mL (Groen et al., 2011).

Problem
There is tubulin in the flow-through.

Potential Solution
This unbound fraction may represent (1) denatured tubulin, (2) saturation of the TOG column or (3) a decrease in binding capacity of the TOG column. The best way to distinguish between these three potential problems, is to load a known amount of tubulin onto the TOG column and analyse how much is recovered.

Problem
You lose most of your tubulin during the concentration step.

Potential Solution
Tubulin concentration should be determined before and after concentrating to determine concentration efficiency and potential protein loss. To minimize non-specific adsorption of protein to the walls and filter, equilibrate the concentration column as described in step 54. Centrifuge the concentration column for no longer than 10 min at a time. Longer spins allow precipitated protein resulting from an increased local concentration above the membrane to build up and block the flowthrough. Carefully resuspend any precipitates, which could contain functional polymerized tubulin, and rinse the membrane three times with the remaining protein solution between spins. At the end of the concentration step, take out the concentrated tubulin and rinse the concentration column including the membrane with a minimal volume of desalting buffer to recover any residual tubulin. Take care at all times to avoid touching and damaging the filter membrane.

Problem
The tubulin yield is much lower than expected.

Potential Solution
This can result from multiple factors. Make sure to use appropriate amounts of starting material. Furthermore, check whether and how efficiently tubulin did bind to the TOG column, which will become evident when doing the quality control. Make sure to not lose most of the tubulin during the concentration step.

Problem
The tubulin does not polymerize using standard tubulin polymerization protocols.

Potential Solution
For tubulin purifications from different species, you might need to test a variety of polymerization methods to find a suitable condition promoting microtubule growth. Critical concentration and temperature are particularly important parameters to consider. For example, at 37°, *X. laevis* tubulin will polymerize at concentrations as low as 1.5 μM, whereas bovine brain tubulin will not. Assembling microtubules in the presence of a microtubule-stabilizing drug such as Taxol will help demonstrate that the tubulin is indeed functional, even if optimal growth conditions have not yet been established. Another problem might be hydrolyzed GTP, in which case use a fresh aliquot of GTP.

Problem

A major portion of the purified tubulin is inactive.

Potential Solution

Soluble tubulin will lose activity over time, even if kept on ice or at 4°C. Take care to avoid delays between steps of the protocol, especially when the tubulin is in the high-salt elution buffer.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Simone Reber (simone.reber@iri-lifesciences.de).

Materials Availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Simone Reber (simone.reber@iri-lifesciences.de). In general, plasmid constructs and antibodies are available for sharing.

Data and Code Availability

This study did not generate any unpublished custom code, software, or algorithm.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.R. and S.R.; Methodology, S.R., A.B., and W.G.H.; Validation, S.R. and W.G.H.; Writing – Original Draft, S.R., A.B., W.G.H., and S.R.; Writing – Review & Editing, S.R., A.B., W.G.H., and S.R.; Funding Acquisition, S.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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