Purification of tubulin

Equipment:

Ultracentrifuges (Beckman Avanti and Beckman Ultra max or equivalent)
Rotors (JLA 8.1000, JA-12, MLA80 or equivalent (1 L tubes, 50 mL tubes, ~6 mL tubes)

Columns/resin:

GSTrap affinity column (or glutathione resin)
HiTrap NHS-activated HP column (1 or 5 mL)
PD10 desalting columns
10K MWCO centrifugal filter (Amicon Ultra or equivalent)

Cells:

BL21(DE3) T1 pRARE E. coli

Reagents:

Ampicillin
Chloramphenicol
IPTG
DTT
L Terrific Broth (or LB… but will yield less recombinant protein)
Benzonase
Protease inhibitors (roche tablet or equivalent)
Potassium phosphate KH_2PO_4
Sodium phosphate Na_2HPO_4,
KCl
ATP
MgCl2
Glutathione (reduced)
HCl
NaCl
Ethanolamine
Sodium carbonate $NaHCO_3$
Glycerol
PIPES
EGTA
GTP
Tween 20
Ammonium sulfate $(NH_4)_2SO_4$

Purification of TOG domains

Stock solutions:

100 mM $NaHCO_3$ with 100 mM NaCl at pH 8.2
10X PBS: 27 mM KCl, 15 mM KH_2PO_4, 81 mM Na_2HPO_4, 1.37 M NaCl
6X PBS
2X PBS
2X PBS with 1 mM DTT and 0.1% tween-20
2X PBS with 5mM ATP with 10mM $MgCl_2$
2X PBS with 1 mM DTT and 5 mM reduced glutathione

1. Transform pGEX-6P-1 Stu2 1-306 or pGEX-6P-1 Stu2 1-590 into in BL21(DE3) T1 pRARE

2. Innoculate an overnight culture in MDAG-135 (or LB) containing antibiotics ((100 µg/mL ampicillin and 15 µg/mL chloramphenicol).

3. Dilute culture 500-fold into 1 L Terrific Broth (or LB) with the same antibiotics and shake at 37°C until the OD600 reaches 0.5.

4. After shifting the cultures to 18°C for 1 hour, induce expression with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubate overnight (18 hours)

5. Pellet induced cultures at 5,000 rpm in a Beckman JLA 8.1000 for 10 min at 4°C)

6. Resuspend in an equal volume of 2X PBS made from a 10X stock (10X PBS: 27 mM KCl, 15 mM KH_2PO_4, 81 mM Na_2HPO_4, 1.37 M NaCl)

7. After adding benzonase (Novagen) and protease inhibitors, lyse cells using two passes through an ice cold Emulsiflex C5 microfluidizer (Avestin) at 1500 bar.

8. Clarify lysate by two sequential centrifugation steps (12 K rpm, Beckman JA-12; 30 min at 4°C) or one 20 min spin at 50,000 rpm in a Beckman ultracentrifuge if volume permits.

9. Apply to a GSTrap (5 mL = 1 column volume, GE Healthcare) pre-equilibrated in 2X PBS with 1 mM DTT (Wash Buffer) at 0.5 column volume (CV)/min.

10. Wash with 10 CV wash buffer with 0.1% Tween20,

11. Wash with 2 CV 5mM ATP with 10mM $MgCl_2$ in 2x PBS and incubate for 20 min

12. Wash with 5 CV of 6X PBS, followed by 5 CV of wash buffer.

13. Elute the GST-fusion using 5 mM reduced glutathione in wash buffer at pH 8.0.

14. Pool eluted protein and desalt with a PD-10 column or dialyze against three changes of 100 mM $NaHCO_3$ with 100 mM NaCl at pH 8.2 (Coupling Buffer).

15. Proceed with conjugation to NHS column

Conjugation with NHS column (modified from protocol from GE healthcare)

Stock solutions:
1mM HCl
0.5 M Ethanolamine, 0.5 M NaCl pH 8.3
6x PBS
1xPBS with 50% glycerol

1. Add MgCl2 to protein solution to 80mM final.

2. Wash out storage buffer with 5 CV ice cold 1mM HCl

3. Immediately load protein in volume of at least 3.5 mL. Allow protein solution to recirculate at 1.5 CV/min on the column for 20 min.

4. Wash with 6 CV 0.5 M Ethanolamine, 0.5 M NaCl pH 8.3 to block remaining reactive groups and incubate for 30 min

5. Wash with 10 CV 6x PBS

6. Wash with 5 CV or 1xPBS with 50% glycerol (0.5 CV/min)

7. Store at -20 C

Affinity purification of tubulin from cultured cells (S2, SF+ or HEK293) on immobilized TOG domains

Stock solutions:

5X BRB80
1X BRB80
Elution Buffer: 0.5 M $(NH_4)2SO4$ in 1X BRB80, 10 μM Mg^{2+}GTP
benzonase (Novagen 70746)
1 M DTT
10 mM GTP
1 M $MgCl_2$
100 mM ATP
Storage buffer: 1X PBS, 50% glycerol
10X PBS (27 mM KCl, 15 mM KH_2PO_4, 81 mM Na_2HPO_4, 1.37 M NaCl)
1X PBS

Affinity purification protocol

1. Pellet 1-3 L cells at high density (500 x g for 10 min)
2. Total weight of cells _____

3. Re-suspend in equal vol. 1X BRB80 to a total volume (cells + buffer) of ____ mL
4. Add 3 μL benzonase and DTT to 1mM final
5. Dounce on ice 20 strokes
6. Centrifuge 80 K rpm S100 AT6, 2°C, 15 min
7. Collect supernatant (__ mL) and, working in the 4°C cold room, load at 0.5 CV /min onto TOG12-Column pre-equilibrated in 1X BRB80.
8. Wash with 4 CV 1X BRB80, 100 μM Mg^{2+}GTP to clear out most of the extract from the column.
9. Change flow rate to 1 CV /min
10. Wash with 10 CV 1X BRB80, 100 μM Mg^{2+}GTP
11. Wash with 3CV 1X BRB80, 10 mM MgCl2, 5 mM ATP 4 CV, 100 μM Mg^{2+}GTP; incubate 15 min
12. Wash with 5 CV 1X BRB80, 100 μM Mg^{2+}GTP
13. 5 CV 1X BRB80, 0.1% Tween 20, 10% glycerol
14. Elute tubulin with 3 CV Elution Buffer (0.5 M $(NH_4)2SO4$ in 1X BRB80, 10 μM Mg^{2+}GTP)
15. Pool tubulin peak by Bradford Assay
16. Desalt into 1X BRB80, 100 μM Mg^{2+}GTP with PD10 columns.

N.B. GTP can be omitted or used at lower concentration (e.g. 10 μM) for GMPCPP assembly

1. Concentrate to at least 20 μM (μL final) and fast freeze in liquid N2.

TOG column cleaning and Storage

1. Clean column immediately after use at 4°C.
2. Set flow-rate to 1 CV/min and wash with 5 CV 1X PBS
3. Lower flow-rate to 0.5 CV/min and wash with 10 CV 10X PBS
4. Wash with 10 CV 1X PBS, 50% glycerol
5. Cap column tightly with fittings. Store capped column in a conical tube at -20°C.