Standard Operating Procedure
for
Hazardous Chemicals

Principal Investigators: Chung-Jui Tsai and Scott A. Harding
Building and rooms: Life Sciences bldg, Lab B310

Chemicals	1,4-Piperazinediethanesulfonic acid (PIPES), Ethylene glycol-bis(2-amino ethylether) N,N,N,'N'-tetraacetic acid (EGTA), Tris-HCl, leupeptin, Phenylmethylsulfonyl fluoride (PMSF), benzamidine-HCl, pepstatin A, GTP, DEAE-Sephadex A50, KCl, NH_4HCO_3, Dithiothreitol (DTT), liquid nitrogen (LN)
Process	**Tubulin protein purification**
Specific hazards	PIPES may be harmful upon skin/eye contact, Tris-HCl is skin and eye irritant and harmful when inhaled, leupeptin-neurotoxin, PMSF-highly toxic, Benzamidine HCl- harmful when in skin/eye contact and upon inhalation, pepstatin A- may be skin/eye irritant, GTP-toxic, irritant to skin/eye/inhalation, KCl-skin and eye irritant, NH_4HCO_3-harmful, DTT-harmful when in contact with eyes/skin and upon inhalation, LN is a frostbite hazard.
Personal protective equipment	Must wear 3-5 mil nitrile gloves. Chemical safety goggles and lab coat should be worn when splash potential exist. In case of insufficient ventilation, wear suitable respiratory equipment.
Engineering/ ventilation controls	: chemical fume hood : emergency shower and eyewash accessible
Special handling procedures and storage requirements	: flammable cabinet, Rm 310 : store away from oxidizers :store away from flammable reagents, under hood in Rm 310 : 4^oC refrigerator, Rm 310 : 4^oC cold room, Rm 313 : -20^oC freezer, Rm 310
Spills and accidents procedures	Skin exposure: Rinse affected skin with plenty of water while removing contaminated clothing/shoes. Rinse for > 15 minutes. Eye exposure: Wash eyes for > 15 minutes. For both cases, seek medical attention immediately.
	Small (< 2L): Absorb with vermiculite or spill pads and transfer absorbed material to a closed container. Label and date as hazardous waste for disposal. Notify PIs.
	Large (> 2L): Evacuate the room, notify PIs and call 2-2200 to request emergency spill assistance from the Biosafety Office.
Waste disposal	Quantities used in most protocols are so small that disposal is not an issue. Significant quantities should be collected and labeled as hazardous waste according to the SOP for Hazardous Waste Disposal.
Special approval	No special authorization needed after SOP training and reading MSDS.
Prepared by	Name/Date: Prashant Swamy/ 09-13-2010
Reviewed by	Name/Date: CJ Tsai / 09-15-2010

Tubulin Protein Purification from Xylem

Chemicals:

1,4-Piperazinediethanesulfonic acid (PIPES)
Benzamidine HCl
DEAE-Sephadex A50
DTT (dithiothreitol)
EGTA (ethylene glycol-bis(2-amino ethylether) N,N,N,'N'-tetraacetic acid)
GTP (Sigma G5884, 25 mg)
KCl
Tris-HCl

Protease inhibitors:

Leupeptin (USB 18413, 25 mg)
NH_4HCO_3
TPCK (N-p-Tosyl-L-phenylalanine chloromethyl ketone, Sigma T4376, 250 mg)
Pepstatin A (Sigma P5318, 25 mg)
PMSF (Phenylmethylsulfonyl fluoride, RPI P20270, 5 gm)
NaF (Sodium fluoride, Sigma S0625, 5 gm)

Stock solutions:

1 M DTT: Dissolve 3.09 g of dithiothreitol in 20 ml of 0.01 M of sodium acetate (pH 5.2) and filter-sterilize. Make 1 ml aliquots and store at -20 ^{0}C.

0.1 M EGTA: Dissolve 0.38 g of EGTA powder in 8 ml of sterile distilled water and adjust pH to 8.0 or until EGTA is dissolved completely. Make up volume to 10 ml, filter-sterilize and keep at room temperature.

1 M $MgCl_2$: Dissolve 20.33 g of $MgCl_2.6H_2O$ in 80 ml of H_2O. Adjust volume to 100 ml and sterilize by autoclaving.

0.1 M PIPES-KOH, pH 6.9: Add 0.302 gm of PIPES to 8 ml of H_2O and adjust the pH to 6.9 with 2N NaOH. Adjust the volume to 10 ml and filter-sterilize. Store at 4^{0}C.

0.1 M Benzamidine-HCl: Add 0.156 gm of benzamidine-HCl to 10 ml of H_2O and filter-sterilize.

100 mM GTP*: Add 480 µl of sterile water to 25 mg vial of GTP, mix thoroughly and aliquot into two 1.5 ml tubes. Store at -20 ^{0}C.

1 M KCl: Dissolve 7.455 gm of KCl in 90 ml of water, make up the volume to 100 ml and sterilize by autoclaving. Store at room temperature.

0.01 M NH_4HCO_3: Dissolve 1.98 gm of ammonium bicarbonate in 50 ml sterile water. Sterilize by filtration and store at room temperature.

10 mg/ml Leupeptin*: Add 2.5 ml of sterile deionized water to 25 mg vial. Dissolve completely, make small aliquots and store at -20 ^{0}C.

1 mg/ml Pepstatin A*: Dissolve Pepstatin A with 25 ml of 10% acetic acid (v/v) in methanol to 1 mg/ml. Make small aliquots and store at -20 ^{0}C.

100 mM Phenylmethylsulfonyl fluoride (PMSF): Dissolve 0.175 gm in 10 ml of ethanol. Make aliquots and store dry at 4 ^{0}C.

100 mM N-tosyl-L-Phenylalanine chloromethyl Ketone (TPCK): Dissolve 0.35185 gm in 10 ml of methanol to get 100 mM concentration. Make 10 mM working concentrations by diluting 1 ml of 100 mM TPCk to 9 ml of methanol. Store in aliquots at -20 ^{0}C.

1 M Sodium Fluoride (NaF): Dissolve 0.42 gm in 10 ml sterile deionized water. Make 1 ml aliquots and store at -20 ^{0}C.

** These chemicals are expensive and most are packaged in small amount. When preparing stock, add water (or appropriate sovent) to the vial, resuspend thoroughly, aliquot, labeled with date and your initials and store at -20^{o}C. **DO NOT** attempt to weight mg of chemicals for stock preparation (we waste more this way).*

PEM Buffer: store at 4^{0}C

	Final	stock	add
DTT	1 mM	1.0 M	50 µl
EGTA	1 mM	0.1 M	500 µl
$MgCl_2$	0.5 mM	1.0 M	25 µl
PIPES-KOH (pH 6.9)	50 mM	0.1 M	25 ml
Sterile water	--	--	to 50 ml

Extraction buffer: add protease inhibitors and GTP to10 ml PEM buffer just before use

	Final	stock	add
PEM	--	--	10 ml
Benzamidine HCl*	1 mM	0.1 M	100 µl
Leupeptin*	2 mM	10 mg/ml	1 µl
Pepstatin A*	15 mM	1 mg/ml	100 µl
PMSF* (in EtOH)	1 mM	100 mM	50 µl
TPCK*	50 µM	10 mM	10 µl
NAF*	1 mM	1 M	10 µl
GTP	2 mM	100 mM	200 µl

DEAE-Sephadex A50 mix

Equilibrate 0.5 g of DEAE-Sephadex A50 to 30 ml of PEM buffer in a falcon tube and mix thoroughly. DEAE will swell and increase in volume. Let DEAE settle and remove the buffer without touching the resins. Add more PEM buffer and mix again to wash the resins. Repeat this step twice.

Wash buffer (make fresh each time)

	Final	Stock	add
PEM buffer	--	--	9 ml
GTP	0.1 mM	100 mM	15 µl
KCl	0.4 M	0.5 M	6 ml

Elution buffer

	Final	Stock	add
PEM buffer	--	--	1 ml
GTP	0.1 mM	100 mM	5 µl
KCl	0.8 M	0.5 M	4 ml

Dialysis buffer (2L)

	Final	Stock	add
NH_4HCO_3	0.01 M	0.5 M	40 ml
Sterile Water			to 2 L

Procedure:

1. Grind the xylem tissue (approximately 5 g) to a fine powder in liquid nitrogen with sea sand, using LN-pre-cooled mortar and pestle.

2. Using a LN-pre-cooled spatula, transfer the fine powder immediately to 2 volumes of extraction buffer in an ultracentrifuge tube (polycarbonate centrifuge bottle, Beckman 355603) (*e.g.* 5 ml of tissue powder volume and 10 ml of extraction buffer) and vortex vigorously.

3. Centrifuge the suspension at 50,000 g for 10 minutes at 2^0C in an ultracentrifuge tube **(Obtain training and permission from Scott or C-J before performing ultracentrifugation)**.

4. Collect the supernatant into a fresh ultracentrifuge tube and ultracentrifuge again at 100,000 g for 45 minutes at 2^0C.

   During the 2nd ultracentrifugation, take an aliquot (= 0.5 volume of the crude protein) of the PEM-equilibrated DEAE-Sephadex A50 in a Falcon tube, and add GTP to a final concentration of 0.5 mM. Mix and store on ice.

5. Save 100 µl of the crude protein (supernatant) for analysis as fraction A. Collect the rest of the supernatant into the Falcon tube containing DEAE-Sephadex A50 and GTP. Mix and incubate the mixture for 1 hr at 4^0C under slow swirling (allowing tubulins to bind to GTP).

6. Apply the incubated mixture to a BioRad polyprep chromatography column (0.8 cm X 4 cm).

7. Collect the flow through (unbound protein) into one separate tube. Save 100 µl for further analysis as Fraction B.

8. Wash the column with 3-5 volumes of wash (low salt) buffer. This will remove loosely bound proteins. Collect into a fresh tube and save 100 µl for analysis as fraction C.

9. Elute tubulins by loading 1 bed volume of elution (high salt) buffer to the column. Collect eluant in separate 1.5 ml tubes, ~500 µl each. Label the fractions as D1, D2, etc.

10. Confirm the presence of proteins in all eluant fractions by the Bradford analysis. Determine the concentration and yield, and pool the fractions containing proteins as fraction D into one tube.

11. Dialyze the pooled fraction D using 1 L of 0.01 M NH_4HCO_3 for 10 hrs at 4 ^{0}C. Replace with fresh 1 L of 0.01 M NH_4HCO_3, dialyzed for 2 hrs more and proceed to the next step.

12. Concentrate the protein using a Nanosep centrifugal column (MWCO 10K) and spin at 14000 xg for 10 minutes. Collect the concentrate in 1.5 ml tube (if membrane appears dry with no buffer in the reservoir, add 20 µl of 0.01 M ammonium bicarbonate to the reservoir, pipette up and down very gently and save the concentrate to a new tube as enriched tubulins). Repeat this procedure until you concentrate all dialyzed fraction D. Save a small aliquot for Bradford analysis and subsequently QC by SDS PAGE.

13. To the concentrated protein, add ethylene glycol to final concentration of 10%. Aliquot and snap-freeze the protein in LN and store at -80 ^{0}C.

14. Confirm the proteins from all fractions by SDS-PAGE using an equal amount of proteins from all fractions. It is necessary to desalt the protein in fraction C by dialysis as in step 12. Alternately, quick desalting can be achieved by buffer exchange using PEM without GTP†.

†Buffer exchange

Mix ~0.5 g of Sephadex G25 to 30 ml of PEM buffer, mix and incubate at 4^{0}C. After resin mix settles, remove the buffer from top and add more PEM. Repeat this twice and store at 4^{0}C until use. To the 5 ml syringe bottom, place small amount of glass wool to stop the resin leak. Add 300 µl of resins to the syringe, place the syringe in a 15 ml Falcon tube and spin at 300 xg for 1 min. Discard the liquid in the tube, re-load the resin at least once and spin again. Add 500 µl PEM buffer (or the same volume as your fraction C) to equilibrate the column at least twice, or until the input volume = output volume. When the column is equilibrated, place it into a new tube, and add add 200-500 µl of protein to be desalted. Spin as before and collect the flow through. Quantify the protein and use for SDS-PAGE.

References

Morejohn, L. C., Fosket, D. E. (1982). Higher plant tubulin identified by self-assembly into microtubules *in vitro*. Science **297** (3): 426-428.

Smertenko A, Blume Y, Viklicky V, Opatrny Z, & Draber P (1997) Post-translational modifications and multiple tubulin isoforms in Nicotiana tabacum L cells. *Planta* 201(3):349-358.