Large Scale Tubulin Preparation

Tubulin is purified from bovine/porcine brain by two cycles of polymerization/depolymerization followed by removal of copurifying proteins on a phosphocellulose (PC) column. The procedure described here is for a large scale prep (10 cow brains) that yields 1-4 grams of tubulin. The protocol can be scaled down if such a large prep is either not necessary or not doable. For ease of organization, all the pre-prep and day-of-prep activities are listed in outline form before details about the prep itself.

I. Tubulin Prep Outline

Pre-Prep:

1. Call slaughterhouse and request fresh brains to be picked up the morning of prep

2. Pour and equilibrate phosphocellulose column

3. Make buffers

4. Ensure that reagents, such as ATP and GTP, are present in sufficient

amount for the prep

5. Sign up for centrifuges and rotors and gather centrifuge tubes,

blenders and motorized homogenizer/dounce

6. Prepare cooler for transporting brains night before prep and organize

coldroom for morning mayhem

Prep Day:

7. Remove meninges, brain stems and blood clots, weigh and

homogenize brains in blenders

8. Clarify homogenate and use supernatant for 1st polymerization cycle

9. Collect 1st cycle polymer fraction by centrifugation

10. Depolymerize 1st cycle polymer by homogenization at 0-40C

11. Clarify depolymerization mix and use supernatant for 2nd

polymerization cycle

12. Collect 2nd cycle polymer fraction by centrifugation

13. Depolymerize 2nd cycle polymer by homogenization at 0-40C

14. Clarify depolymerization mix and load supernatant onto PC

column

15. Collect flowthrough from PC column, aliquot and freeze at -800C

II. Buffers & Nucleotides

PB (Pipes/Polymerization Buffer): 0.1 M K-Pipes, pH 6.8, 0.5 mM $MgCl_2$, 2 mM EGTA, 0.1 mM EDTA, 0.1 % b-mercaptoethanol, 1 mM ATP. Need 8 liters in coldroom (Add ATP and BME just prior to beginning the prep) CB (Column Buffer): 50 mM K-Pipes, pH 6.8; 1 mM EGTA; 0.2 mM $MgCl_2$. Need ~25 liters for equilibration, running and storage of PC column CB + 1 M KCl: Need ~10 liters for prewashing and eluting the PC column To make 1L of 10X CB: 151.2 grams PIPES, free acid 3.8 grams EGTA 2 ml of 1 M $MgCl_2$ pH with KOH to pH 6.75, and bring up to 1 liter. Check pH at 1X is 6.7 Make 3.5 liters of 10X CB for 10-12 brain prep. GTP: Sigma Type IIS- # G-8752 ATP: Sigma Grade 1- # A-2383 Glycerol: 2-3L prewarmed to 37iC (store overnight in 37iC incubator)

III. Pouring a 1L Phosphocellulose (PC) Column

Resin: Whatman P11 Cellulose Phosphate -- fibrous cation exchanger (1 gram of PC swells to about 4 ml packed

resin) Summary: To pour a 1L column, start with 220 grams dry resin divided into 5 aliquots of 44 grams. Treat each aliquot with acid/base in a 2L beaker as described below. Older procedures described the use of large Buchner funnels to rapidly remove the acid/base. However, gentle stirring of the resin with a plastic/glass rod to suspend it in a 2L beaker, followed by settling of the resin for 5' by gravity has worked well for us. This method also incorporates de-fining of the resin into the acid/base cycling protocol. After acid/base treatment the resin is washed well, packed, treated with BSA to block irreversible binding sites and equilibrated for use. Solutions & Supplies: 220 grams Phosphocellulose 5L 0.5N NaOH 5L 0.5N HCl 13L 0.5M K-Phosphate, pH 6.8 5L ddH_20 5 2L beakers 12L CB + 1M KCl 20L CB 300 ml of 30 mg/ml BSA in CB (filtered) 1-1.5L cleaned column housing 2 stirring rods 2 aspirators with large traps Peristaltic pump 10 ml plastic pipets as inlets for peristaltic pump

Column Preparation Procedure: **1.** Pour 1L 0.5N NaOH into 5 x 2L beakers. Add 44 grams PC to each beaker stirring gently with a rod until the PC is wetted and an even slurry is present. Let stand at room temperature for 5'. **2.** Aspirate off supernatant, including fines, and quickly add 1L 0.5M K-phosphate to neutralize, gently mixing with a rod. Check that pH is ~7 and let stand 5'. **3.** Aspirate off supernatant, add 1L ddH_20 and gently stir to resuspend settled resin. **4.** Allow the resin to settle. **5.** Aspirate off supernatant, add 1L 0.5N HCl, gently stir to resuspend and wait 5'. **6.** Aspirate off supernatant, add 1L 0.5M K-phosphate, stir and check pH is 7. **7.** After resin has settled, aspirate supernatant and combine all the resin in a 4L beaker. Use the remaining 0.5M K-phosphate to wash the resin by resuspending, letting settle and aspirating the supernatant. **8.** Wash 3 x 1L CB + 1M KCl as done in 7. **9.** In the cold room, pour the resuspended resin into the column housing (with a mark approx. 50 cm high in a 5 cm ID housing) and pack by pumping from the bottom (i.e. the peristaltic pump is "sucking" buffer from the bottom of the column and depositing it into a waste jug). Pack at 45 ml/hour/cm cross-sectional area. For a 5 cm diameter column this is ~880 ml/hour or ~14.5 ml/min. After resin is packed, switch to pumping from the top. Run 7L of CB + 1M KCl through the column at 5-10 ml/min. **10.** Wash with 10L CB. Check conductivity to ensure that all the KCl is gone. The resin may expand as the salt is washed out so make sure there is a large buffer head on the resin bed. **11.** Load 300 ml of 30 mg/ml BSA (Fraction V; filtered) in CB, follow with 700 ml CB and stop the column. Leave the column sitting for 2 hr during which the BSA blocks irreversible binding sites on the resin -- this is very important the first time a column is used to prevent loss of the tubulin. **12.** Wash the

column with 2L CB + 1M KCl to elute BSA that is not irreversibly bound. **13.** Wash column with 10L of CB. The column is now ready for use.

IV. Brains

It is essential to get fresh brains (they should be warm when handed to you at the slaughterhouse) -- yields decline significantly if the brains have been stored for a while after removal. Frozen brains do not work for preparing tubulin. The best preps have been done with freshly removed brains transported in an ice-filled cooler to the lab within 1-2 hours of removal. For transporting 10-12 brains from the slaughterhouse, we use 2 size 16 Coleman coolers containing 3 liters of cooled 1.5% (w/v) NaCl to which one large bag of party ice is added on the way to the slaughterhouse.

V. Centrifuges & Rotors

6 Sorvall RC-5C or equivalent lowspeed centrifuges

6 GSA or equivalent rotors (cold)

4 Beckman ultracentrifuges

4 Type 19 rotors (warm)

2 Type 35 rotors (warm)

2 Type 45Ti rotors (cold; need to warm up one after 1st cold use)

1 Type 50.2Ti rotor (cold)

cold = 4iC (put overnight in cold room)

warm = 37iC (put the rotors overnight in a large bacterial shaker set to 37iC)

VI. Protocol

1. In the cold room, remove meninges (membrane surrounding the brain; best done by using paper towels to "blot" the brain surface), blood clots, and brain stems; weigh the brains and homogenize with

equal volume of PB for 3 x 15s in a Waring blender. **2.** Collect homogenate (~8-9 liters), transfer into 36 GSA bottles and spin 90' at 12K in a GSA rotor at 4iC. **3.** Collect supernatant and transfer 1 liter to a 1.8L glass Fernbach flask that has 500 ml of 37iC glycerol. Add 0.1 mM GTP, 0.5 mM ATP, and 3.5 mM $MgCl_2$ (this gives 0.1 mM GTP, 1.5 mM ATP and 4 mM $MgCl_2$ final). The ATP and GTP are added as solids. Hold the flask in a warm water-filled sink and swirl constantly to dissolve the solids and to mix in the glycerol. Transfer to a 37iC bath, monitor temperature of mixture using a clean thermometer and polymerize for 60' after the temperature of the sample has reached 32iC. The approach to 37iC can be accelerated by swirling the flask in a large hot water (~50iC temperature) reservoir -- constant swirling is essential in this case to disperse the heat evenly and care must be taken to avoid overheating the mixture. **4.** Transfer the polymerization mixture to Type 19 bottles, and spin for 2.5 hrs at 19K in 4 Type 19 rotors at 35iC. Use an additional Type 35 at 17.5K for 2.5 hrs if necessary. At the end of the spin set centrifuges to 4iC. **5.** Decant and discard supernatant. In the cold room, resuspend the gelatinous pellets in PB aiming for a final volume of ~700-800 ml. We use ~40-50 ml for 3 tubes, sequentially removing the pellets from each tube using a plastic scraper and making sure that all tubes get rinsed once or twice after the majority of the large gelatinous pellet has been removed. To homogenize the chunky pellet resuspension, we use a Yamato "pour-through" continuous flow homogenizer -- this is a device that drives a motorized teflon pestle in a funnel shaped glass barrel. Mixtures poured on top get homogenized by the pestle as they travel through the middle of the barrel and come out the bottom. We Yamato the chunky pellets till the resuspension is a smooth yellow liquid of ~700-800 ml total volume. After all the pellets are homogenized, we depolymerize on ice for ~30' during which we continue Yamatoing the mixture once every 3'-5'. A large motorized teflon dounce or large tip sonicator can be used as alternatives to the Yamato for resuspending the chunky pellets. Check protein concentration by Bradford using BSA as a standard. If >20 mg/ml (which is unlikely), dilute to 20 mg/ml. **6.** Spin the depolymerization mixture 30' at 35K in 2 Type 45 rotors at 4iC. At the end of the spin set the centrifuges to 35iC. **7.** Decant supernatant into a 1L graduated cylinder in coldroom and measure volume. Pour into a 1.8L Fernbach flask, add half volume of 37iC glycerol, solid GTP to 0.5 mM final and $MgCl_2$ to 4 mM final (additional 3.5 mM). Set up polymerization as described in **3.** above. Polymerize for 40' after temperature of mixture has reached 32iC. **8.** Spin the polymerization mixture at 35 K at 35iC for 1 hr in 2 Type 35s + 1 Type 45. Make sure one chilled centrifuge is available for the next spin. **9.** Discard

supernatant and resuspend pellets in a final volume of ~100-150 ml of CB, as described above in **5.** Protein concentration by Bradford should not be more than 25 mg/ml. Depolymerize on ice for 40' and then spin the depolymerization mix 30' at 40K in a 50.2Ti rotor at 4iC. **10.** Collect supernatant, measure concentration by Bradford and load onto the PC column (approx. 50 cm high X 5 cm ID = ~ 1L; Flow rate = 6 ml/min). After the sample is loaded and ~150 ml buffer has flowed through start collecting fractions. The eluted tubulin will be apparent by its slight yellowish tinge. Measure concentration by Bradford using BSA as a standard and pool such that the final concentration is between 5-10 mg/ml. Mix pool on ice, make 3 ml aliquots in 5 ml snapcap polypropylene tubes and freeze in liquid nitrogen. Store frozen aliquots at -80iC. The entire procedure, from time of arrival of brains till freezing of the tubulin will take ~17-18 hours. The next day, run 3 volumes of CB + 1M KCl to elute MAPs from the PC column (these can be collected if desired), and then equilibrate column into CB + 0.1% azide for storage. Phosphocellulose will lose capacity when stored wet -- this can be reduced by storage in a phosphate buffer (50 mM phosphate, pH 7 with 1 mM EGTA and 0.2 mM $MgCl_2$) containing 0.1% azide.