Tubulin Purification from Bovine Brain

Dogic Lab – Adapted From Popov protocol.
By: Stephen J. DeCamp. Updated July, 2014.

Reference:
Castoldi M, Popov, AV. Purification of brain tubulin through two cycles of polymerization-depolymerization in a high-molarity buffer. Protein Expr Purif. 2003 Nov;32(1):83-8.

We follow the Popov protocol, but have broken down the purification into step-by-step instructions.

This protocol uses either bovine or calf brains (or porcine brains) and a high molarity PIPES buffer as well as two cycles of tubulin polymerization/Depolymerization (recycling) to purify tubulin protein from brain matter. The protocol efficiently elutes MAPs and can be run in a single day (~10+ hrs).

Red Text are notes made after a prep conducted on:
07-09-2014 Prep Notes

Note: Look ahead through protocol to ensure that centrifuge rotors and buffers are **pre-cooled** to 4C or **pre-heated** to 37C prior to the step they are needed.

Note: Ensure Centrifuge tubes are filled at each step according to their spec.

Reagents and Buffers List

PBS buffer Used ~2L of this to suspend brains.
20mM Na-phosphate + 150mM NaCl
pH 7.2

DB – Depolymerization Buffer Used ~1L of this total
50mM MES (2-[N-morpholino]ethanesulfonic acid) + 1mM CaCl2
pH 6.6 with HCl

HMPB – High Molarity Pipes Buffer Used ~1L of this
1M PIPES + 10mM MgCl + 20mM EGTA
pH 6.9 with KOH

100mM ATP in water, pH 7.5 with NaOH

200mM GTP in water, pH 7.5 with NaOH

Warm Glycerol. Used ~0.5L of this

Reagents and Buffers Prep Instructions

- **PBS buffer**

  20mM Na-phosphate + 150mM NaCl
  pH 7.2 with NaOH
  Need for the storage of the brain tissue during transport from a slaughterhouse.

  For V = 1L (approx.)
  *m(NaH_2PO_4) = 20 mM * 138 g/mol = 2.75g*
  *m(NaCl) = 150 mM * 58.4 g/mol = 8.75 g*

- **DB – Depolymerization Buffer**

  50mM MES (2-[N-morpholino]ethanesulfonic acid)
  1mM CaCl2
  pH 6.6 with HCl
  Need 1 L per kg of the brain tissue.

  For V = 1L
  m(MES) = 50 mmol 195.2 g/mol = 9.76 g (per L)*
  *m($CaCl_2$) = 147 g/mol * 1 mM = 0.15 g (per L)*

- **HMPB – High Molarity Pipes Buffer**

  1M PIPES + 10mM $MgCl_2$ + 20mM EGTA
  pH 6.9 with KOH
  Need to mix it with supernatant at 1:1 ratio, so max necessary volume is estimated to be 2L per kg of brain tissue (1L DB + ~1L tissue : 2L HMPB)

  *m(PIPES) = 1 M PIPES = 1 mol/L * 302 g/mol = 302g/L*
  *m($MgCl_2$) = 10 mmol/L * 203.3 g/mol = 2.03 g/L*
  *m(EGTA) = 20 mmol/L * 380.35 g/mol = 7.6 g/L*

  Note: *This buffer will require a LOT of KOH.*

- **100mM ATP in water, pH 7.5 with NaOH**

  This stock solution will be added to HMPB-Supernatant mixture to final concentration of 1.5 mM.

  To make the stock solution
  *c(ATP) = 100 mM *551.14 g/mol = 55 mg/mL*
  *m(ATP) = 61 mL/kg brain * 55 mg/mL = 3.3 g/kg*
  Make ~200 mL of the stock – should be enough for huge brain.

- **200mM GTP in water, pH 7.5 with NaOH**

  This stock solution will be added to HMPB-Supernatant mixture to final concentration of 0.5 mM. This stock will also be used in subsequent polymerizations.

  To make the stock solution
  *c(GTP) = 200 mM *523.18 g/mol = 104.6 mg/mL*
  *m(GTP) = 10 mL/kg brain * 104.6 mg/mL = 1 g/kg*

  Make ~100 mL of the stock – should be enough for humongous brain.

Retrieve Brains
Get brains from slaughterhouse. Place brains directly into ice-cold PBS buffer in gallon freezer bags. Transport in cooler on ice. It is vital to have brains go from 37C to ice as fast as possible. Rush back to Brandeis.
Den Besten Farms (508-697-6500) http://denbestenfarm.com/
Quick from live cow to brain on ice. Slow between cow slaughtering.
Got 2 cow brains. One was small ~350g. The other was larger ~420g.

Brainwashing
In 4C cold room, pat dry the brains with paper towel. Peel off any sinewy, white membrane material on the surface of the brain. Be sure no blood or blood clots remain on the brain. Keep brain cold while handling.
Weigh brains.
Done in Goode Lab cold room.
We had ~660mg of brain after washing and removal of fatty/spinal tissue.

Puree brain
Put brain in blender with ~1L/Kg of DB. Blend for ~30-60 seconds or until smooth. One can get away with less than 1L/Kg to conserve volume for centrifugation step.
Done in Goode Lab cold room
After adding brains into blender, we added enough buffer to cover the brains (not more than 0.5L of buffer was used). The total volume of the brains and buffer was about 1L total! So the 1L/kg estimate is overkill. Final volume of buffer used was likely near 300ml.

1st Spin: Pellet Brain Garbage. (COLD)
Have rotor chilled to 4C.
Pour brain slurry into 500mL tubes for Beckman JA-10. Ensure bottles are weight-balanced.
Spin at 10,000rpm for 60-120 min.
Pellet will be very soft. Avoid agitation. Avoid pouring out any of the pellet.
Done in Goode Lab. They have a Sorvall rotor that holds 4L at similar rcf. In reality, this was volume overkill as the total volume was only 1L. JA-10 holds 3L.

Polymerize MTs (WARM)
Recover supernatant. We did this step in the Goode Lab and transported the supernatant in a bottle back to our lab to polymerize. The pellet was super soft.
We recovered about 300ml of supernatant out of 660mg of brain and 1L of brain puree. The supernatant is pinkish/red.
Add equal volume of warm (37C) HMPB. Added 300ml of HMPB
Add ATP (1.5mM final) and GTP (0.5mM final).
We added 15ml of 100mM ATP, and 2.5ml of 200mM GTP.
Add equal volume warm (37C) Glycerol (1/3 total volume). Added 300ml of glycerol.
Inclubate at 37C for 1 hr.
The mixture immediately turned turbid after adding the GTP and ATP and glycerol. After this, the mixture remained visually the same for the 1hr incubation.

2nd Spin: Pellet MTs (WARM)
Pour polymerized MTs into 70mL tubes for Beckman Ti 45 rotor. Pre warm rotor. Ensure the tubes are filled to the top.
Spin at 151,000g (44,000rpm) for 30min at 37C.
Repeat in same tubes until all MTs are pelleted (if needed).
We completed 2 rounds of this pellet step, and had enough polymerized mixture leftover to simultaneously run a small batch at 28,000rpm in the JA30.50. All tubes showed large pellets with minor bits of junk visible (not completely translucent).
We rinsed all the pellets 2x or 3x with 5ml of warm HMPB only after last run.

Resuspend and Depolymerize MTs (COLD)
Add 100mL of cold DB (~20mL each tube) to resuspend the pellets of MTs.
Leave on ice for 30min.
Added 20ml of DB to each tube. Had 160ml total amongst all the tubes.
We used the 10ml tips on electric pipetters to agitate the pellets… this worked well.
Resuspension was quick and easy.
Put the rotor in the -20 to quickly cool it.

3rd Spin: Clarification Spin (COLD)
Pool resuspended pellets into few tubes to fill centrifuge tubes.
Clarify in Ti45 rotor at 70,000g (30,000rpm) for 30min at 4C. Have rotor chilled.
We filled 3 tubes, ~60ml each.
Alarmingly large pellets were visible after this clarification step.

Polymerize MTs (WARM)
Mix recovered supernatant with equal parts warm HMPB, ATP, GTP, and Glycerol as before.
Incubate at 37C for 30min.
Pooled the supernatants into a 0.5L bottle and added 150ml warm HMPB.
6.76mL of 100mM ATP and 1.125ml of 200mM GTP
Added glycerol to a total volume of ~400ml. This was so we could fit the total volume into 6 tubes for one pelleting spin (not needing to do multiple runs as before).
We placed the rotor in a bag and left in sink with hot water running to warm to 37C.

4th Spin: Pellet MTs (WARM)
Pellet with Ti 45 rotor at 151,000g (44,000rpm) for 30min at 37C. Have rotor warmed.
We rinsed the pellets once again, 2 or 3x with warm HMPB.

Resuspend and Depolymerize MTs (COLD)
Depolymerize MTs, resuspend pellets in 15ml of ice-cold BRB80 and leave on ice for 30min.
We added 3ml of cold BRB80 to each tube (6 tubes total). We used 1ml pipetters to break up and resuspend the pellets. We wound up with ~21ml of total volume.

5th Spin: Clarification Spin (COLD)
Clarify in Ti90 rotor at ~104,000g (35,000rpm) for 30min at 4C.
Small pellets remained, but this is normal.
Recovered 21ml of total volume.

Finish (COLD)
Measure tubulin concentration using Spec.
Dilute to desired concentration.
Freeze in 500uL Aliquots (or desired volume).
Store in -80C freezer for years.

The spec showed the post-clarification concentration to be ~8.5mg/ml.
This was aliquoted w/o dilution into 500uL aliquots. We got a total of 42 aliquots at 8.5mg/ml.
The total yield was 180mg of tubulin from 660g of brain.
This is roughly consistent with the prep that produced the previous lab stock of tubulin.

Popov protocol says optimal is ~650mg from 1kg of brain.
We achieved ~50% of this optimized expected yield.

SDS-PAGE Gel Lanes from 070914 prep By: Feodor Hilitski.
1 - BioRAD broad range standard;
2 - Supernatant after the first spin (brain smoothie);
3 - Supernatant from the first polymerization, after the second spin;
4 - Pellet from the first polymerization resuspended in DB;
5 - Clarification of resuspended pellet;
6 - BioRAD broad range standard;
7 - Supernatant from the second polymerization (after spin);
8 - Pellet from the second polymerization resuspended in BRB80; estimated concentration ~8.5 mg/ml of tubulin;
9 - Clarification of the pellet, dulitet 1:10 in BRB80; estimated ~8 mg/ml of tubulin (before dilution);