Tubulin Protein Recycling Guide

Dogic Lab – Adapted from Mitchison Lab Protocols
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Tubulin recycling is a process which further purifies polymerization-competent tubulin from polymerization incompetent tubulin which can aggregate and dirty samples. The protocol cycles tubulin into microtubules and then depolymerizes back to tubulin, with intermediate centrifugation steps to eliminate excess defunct protein.

This guide is for recycling 1ml of tubulin suspended in BRB80 or M2B buffer.
ie: 2 aliquots of 7.4mg/ml tubulin (0.5ml each) obtained from Bovine Brain.

Materials to Prep:

BRB80: (80mM K-Pipes, 1mM MgCl2, 1mM EGTA, pH 6.8 with KOH)
We often substitute M2B for BRB80 as all of our experiments are conducted in M2B buffer.

Cushion: 60% (v/v) glycerol in BRB80
Typically just mix 3ml of Glycerol with 2ml of BRB80

M2B: (80mM K-Pipes, 2mM MgCl2, 1mM EGTA, pH 6.8 with KOH)
~20ml of M2B, filtered

DTT: 0.5 M in M2B (pH 6.8)
Only need 1ul

GTP: 100mM in M2B (GTP = 523.18g/mol)
2.6mg GTP into 50ul of M2B

Notes:

Have in Hot bath:
Turn on Hot bath. Equilibrate to 37C.
Put M2B (at least 12ml) in hot bath for warm rinsing the pellet.
Put Ultracentrifuge Rotor in hot bath to equilibrate to 37C

Have on Ice:
Put ~1.5ml of M2B on ice for de-polymerizing and suspending tubulin.
Put a few 0.5 and 1.5ml tubes on ice to be equilibrated and cold.

This protocol is compatible with either of the following centrifuge rotors:

	Pellet MTs Spin	**Clarification Spin**	**Tube**
TLA-100.4 rotor	30 min @ 80,000 RPM	10 min @ 80,000 RPM	3.5ml Polycarbonate
Type 90 Ti rotor	40 min @ 47,000 RPM	Not yet calculated	4ml Polycarbonate

Step #1: Polymerize MTs (30min)

Combine 2 aliquots of tubulin (1ml total) into a 1.5ml eppendorf tube.
Add ~1ul of DTT stock
Add 20ul of GTP stock
Mix with pipette very thoroughly 20-30 times.
Put on 37C water bath for **30 min**.

Prepare warm cushion and rotor.
Put 1ml of cushion into a centrifuge tube.
Place tubes into rotor and leave in 37C water bath.
Turn on Ultracentrifuge and set to 37C.

Step #2: Pellet the Microtubules (30min)

Transfer MTs into the centrifuge tube. GENTLY lay the MTs onto the cushion.
Ensure the counterweight tube is equivalent.
Place rotor into ultracentrifuge and spin according to **Pellet MTs Spin** in chart.
Spin at 37C

Step #3: Rinse the Interface, Tube Walls, and Pellet

Remove the supernatant above the cushion.
Rinse the tube walls and cushion interface with 2ml of warm M2B 2X changing tips each time.
Remove the supernatant/cushion
Rinse the tube walls and pellet with 2ml of warm M2B 2X or 3X changing tips each time.

Be sure to pipette on the far side of the pellet so as not to disturb the pellet.

*One should be able to just dump the entire supernatant in the garbage and skip the rinsing of the interface as this is mainly important when labeling, however, the above is what I did today.

Step #4: De-Polymerize the Microtubules (30min)

Add 150ul of cold M2B to the pellet.
Keep the pellet covered by the M2B. Keep the tube on ice.
Using a cut tip, mix the M2B every 2-5 minutes.
After pellet thins ~15min, switch to a non cut tip mixing every 2-5min.
Total De-Polymerization time should be about **30 min**.

It is important to minimize bubbles.
Also, keep the tip submerged and partially loaded with M2B so you do not generate air bubbles when replacing the tip onto the pipette each time.

Prepare rotor for cold spin by placing it into the fridge or freezer depending on time.
Turn Ultracentrifuge to 4C.

Step #5: Clarification Spin (10min)

Once the pellet is de-polymerized, place the centrifuge tube into the cold rotor.
Ensure the counter weight is equal (~150ul).
Place rotor into ultracentrifuge and spin according to **Clarification Spin** in chart.
Spin at 4C

Prepare spec for measuring the tubulin concentration.
Dilution is 2ul of tubulin into 98ul of M2B.
Prepare dilution (put 98ul of M2B into cold tube).
Blank Spec with 100ul of M2B

Step #6: Measure Concentration and Aliquot

Transfer supernatant out of centrifuge tube, pipetting away from soft, faint garbage pellet.
Roughly measure volume with pipette
Mix and homogenize supernatant with pipette.
Dilute 2ul into 98M2B and measure Concentration.

Dilute supernatant accordingly, mix with pipette.

Aliquot into micro tubes.
Freeze in LN2.
Place in -80C