Tubulin Protein Labeling Guide

Dogic Lab – Adapted from Mitchison Lab Protocols
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This guide is for recycling 6ml of tubulin (12 aliquots of 7.4mg/ml tubulin stock, 0.5ml each). This is a HUGE prep that requires a large amount of expensive Alexa Dye. It can easily be scaled down to label smaller amounts of tubulin.

Materials:

Dye Stock: Alexa Fluor 647 Carboxylic Acid, Succinimidyl Ester (A-20006)
https://products.invitrogen.com/ivgn/product/A20006?ICID=search-product
MW = ~1300g/mol

High pH Cushion: 0.1M NaHEPES, pH 8.6, 1mM MgCl2, 1mM EGTA, 60% (v/v) glycerol

Labeling Buffer: 0.1M NaHEPES, pH 8.6, 1mM MgCl2, 1mM EGTA, 40% (v/v) glycerol

Quench: 2x BRB80, 100mM K-Glutamate, 40% (v/v) glycerol

Low pH Cushion: 60% (v/v) glycerol in M2B

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

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

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

This protocol is compatible with either of the following centrifuge rotors:
* Ensure the Centrifuge Tubes are filled or spin at lower speed according to specifications.

	Pellet MTs Spin	**Clarification Spin**
TLA-100.4 rotor	30 min @ 80,000 RPM	10 min @ 80,000 RPM
Type 90 Ti rotor	40 min @ 45,000 RPM	Not yet calculated

Step #1: Polymerize MTs (30min)

Combine 12 aliquots of tubulin (6ml total) into one 15mL tube.
Add ~6ul of DTT stock to each tube
Add 100ul of GTP stock to each tube
Mix with pipette very thoroughly 20-30 times.
Put on 37C water bath for **30 min**.

Step #2: Pellet the Microtubules (30min)

Put 1ml of warm High pH Cushion in centrifuge tubes.
Transfer MTs onto the cushion in the centrifuge tubes. Use 2 tubes, 3ml of MTs each.
Ensure the counterweights are there.
Place rotor into ultracentrifuge and spin according to **Pellet MTs Spin** chart at **37C**

Step #3: Rinse Pellet and Resuspend

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

Resuspend the pellet in 1.4ml of warm labeling buffer (0.7ml in each tube).
Keep pellet warm while pipetting the pellet off of the tube.

Once resuspended, combine into one tube.

Step #4: Label MTs (30min)

Add 10-20 molar excess of dye. Figure 70% recovery rate per polymerization.

Use 4mg of Alexa 647 (4x 1mg tubes).
Add 70ul of DMSO for each 1mg of dye to the dye stock. Suspend dye by vortexing.
Add 280uL of dye to MTs.
Vortex MTs gently every 2-3min. Keep the MTs at 37C.
To reduce bubbles use an ependorff tube or a conical tube. Do not use dye tube.

Step #5: Quench Dye (5 min)

Add an equal volume of quench buffer to the labeling mixture and vortex, incubate for 5 min.

Step #6: Pellet the Microtubules (30min)

Put 1ml of warm Low pH Cushion in centrifuge tubes.
Transfer MTs onto the cushion in the centrifuge tubes. Should just need 1 tube.
Ensure the counterweights are there.
Place rotor into ultracentrifuge and spin according to **Pellet MTs Spin** chart at **37C**

Step #7: Rinse Pellet and Depolymerize (30min)

Remove (aspirate) 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.

Resuspend and Depolymerize the Pellet in 0.7ml of cold M2B.
Use a Cut-off tip to break up the pellet. After 15 min switch to normal tip.
Keep tube on ice to promote depolymerization.

Step #8: Clarify Tubulin (10min)

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

Step #9: Polymerize (30min)

Recover supernatant from cold spin. Add 10x M2B to 1x. (ie. 70ul)
Add 5ul of DTT stock.
Add 85ul of GTP (100mM stock)
Put on 37C for 30min.

Step #10: Pellet the Microtubules (30min)

Put 1ml of warm low pH Cushion in centrifuge tubes.
Transfer MTs onto the cushion in the centrifuge tubes.
Ensure the counterweights are there.
Place rotor into ultracentrifuge and spin according to **Pellet MTs Spin** chart at **37C**

Step #11: Rinse Pellet and Depolymerize (30min)

Remove (aspirate) 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.

Resuspend and Depolymerize the Pellet in 450ul of cold M2B.
Use a Cut-off tip to break up the pellet. After 15 min switch to normal tip.
Keep tube on ice to promote depolymerization.

Measure Pre-Clarification Tubulin and Dye concentration.

Step #12: Clarification Spin (10min)

Once the pellet is de-polymerized, place the centrifuge tube into the cold rotor.
Ensure the counter weight is equal
Place rotor into ultracentrifuge and spin according to **Clarification Spin** chart 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 #13: 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.

Freeze in LN2.
Place in -80C