

Yeast Tubulin-6xHis Prep

Harvest cells, store overnight in H-PEM (about 500g cells in 100ml H-PEM).
At start of prep, bring total H-PEM to 700ml.
Make 5mM DTT, 2mM PMSF, 0.1mM GTP, and add Protease Inhibitors (as per Davis paper).
Homogenize cells with 10 passes in Microfluidizer.

Notes: I found storing the cells overnight in ice cold buffer depolymerizes most of the polymer. Also, I have done the prep with only PMSF and no other protease inhibitors and it seems to work about as well. 10 passes in our microfluidizer gives >95% breakage. Also, I found the 2mM EGTA to be vital for DE52 binding. 1mM works but no EGTA and the tubulin doesn't bind.

Spin 10,000xg for 10 min. (about 1/2 volume will pellet)
Spin 100,000xg for 60 min. (High Speed Lysate about 750ml @ 15-25 mg/ml)
This pellet is loose, pour off super but leave the clump of loose pellet behind.
Make Lysate 10% Glycerol and 0.16M NaCl
Add DE52 Slurry (~70%) equil. in H-PEM + 0.16M NaCl. (about 400ml bed)
Stir gently 45 min @ 4C.

Spin down DE52-Lysate slurry (8,000xg for 10 min). (Big rotor as possible)
Wash 2 times with 600ml PMG (NO EGTA) + 0.16M NaCl + 0.1mM GTP
Resuspend DE52 with stirring rod and mix gently.
(spin down as before between washes; be careful, it can be a loose pellet)
Resuspend and pour into column (5.5x30 cm) using 300ml of same wash buffer.
After column is packed, allow about 50ml more wash buffer to pass over DE52.

Notes: It is critical to switch to NO EGTA in these wash buffers now because it will chelate Ni from the polyhis resin and destroy binding. However, as stated, you need EGTA in the homogenization buffer to bind DE52.

Elute column with 750ml 25-PMG (NO EGTA) + 0.5M NaCl + 0.1mM GTP.
Protein will elute after about 1 void volume.
Monitor elution with Bradford Assay.
(don't use O.D. 280 because GTP absorption will mask the protein peak)
Pool fractions containing protein. (Usually about 300ml @ about 0.5-1 mg/ml)
Be sure to collect all trailing fractions of the protein peak because the tubulin elution profile lags behind the major protein containing fractions. These trailing fractions are mostly tubulin. I run the column at full gravity speed (4.5 ml/min) and collect 8 ml fractions.

Sometimes I drop freeze at this step.

Notes: We are using 25mM Pipes at this point because of the aggregation and/or polymerization at the end of the prep. We are concerned that higher Pipes concentration may bring some Ni off the resin. It hasn't produced much better

results, but it doesn't hurt either. The other precaution is to wash the Ni-NTA resin twice with 50 ml loading buffer as below.

Make pooled fractions 20mM Imidazole (I use a 500mM stock buffer), adjust pH to 7.4 (pH will probably work fine at 6.9 but I haven't had the guts to try it). Wash 5 ml Bed Ni-NTA resin (Qiagen) 2 times with 50 ml 6xHis Loading buffer. Resuspend in 5 ml 6xHis Loading buffer and add to pooled fractions. Stir gently 45 minutes @ 4C. Spin down in 50ml falcon tubes and combine resin. (3000xg for 7 minutes). Resuspend in 25 ml 6xHis Binding buffer, mix gently 10 minutes @ 4C. Spin down, wash again with 15 ml 6xHis Wash buffer. Spin down, use about 3 ml 6xHis Wash buffer to load into column. (0.6x30 cm) Let column settle and then slowly run out buffer.

Notes: I have found that the max his-tubulin binding capacity of both the Ni and Co resins is 1.1 mg/ml resin. This is in contrast to the adds stating 5-10 mg/ml. I use 5 ml bed. This will capture 5.5 mg max. If you cut this to 3 ml, you will bind 3.3 mg max, but what you get will not be as dilute coming off.

Elute column with 25 ml 6xHis Elution buffer. (slowly, about 0.5 ml in 3-4 min). Collect small 0.5 ml fractions to prevent excessive dilution of product. Monitor protein elution with Bradford. Pool fractions with protein. It is best to omit fractions with low tubulin conc. as concentrating is the most vulnerable step. I usually draw the line at around 0.5 mg/ml.

Notes: We have been leaving GTP out of the poly-his washing and elution because we are trying to prevent loss due to polymerization at the end when we raise the conc. Also, we don't have glycerol, partly because of that reason but also we don't want it in our rxns.

At this point you should have 2-4 mg of purified tubulin. The max binding of 6xHis tubulin to Ni-NTA resin is 1mg/ml of bed.

Dialyze against PEM buffer in a 0.5-3 ml Slidalyzer Cassette (10K cut-off) from Pierce to remove the imidazole. These are very nice and don't bind much yeast tubulin. They are well worth the couple bucks.

If the conc. is acceptable after dialysis, you can drop freeze at this point without further concentrating.

Concentrating yeast tubulin is the most difficult step in the protocol. I have had some success with 2 methods. The method I prefer right now is keeping the tubulin in the dialysis cassette from above and placing it in Pierce's Slidalyzer Concentrating Solution. This method seems to be pretty efficient because it is rapid and you don't lose a lot of protein in the dialysis cassette. It has drawbacks in that it is hard to monitor the progress and if you go too far it will cut the volume so drastically that you will lose a lot of protein which sticks in the cassette or aggregates. If I put 3 mls in, I would definitely watch the volume closely after 30 min or so. You have to kind of guess how much volume is in

there even if you remove it from the conc. soln. When you want to remove the protein, you have to rinse the cassette thoroughly, so be sure to chill enough dd-H₂O. This method is very fast and efficient. It will cut the volume at least as fast as shown in the pamphlet for the product. You must watch it every 10 min or so and have some idea of the starting and final conc you are aiming for. Also, when the conc goes up and you have to rinse the cassette off prior to extraction, it is A LOT easier to work in a cold room and have everything cold. It is easy to warm up the solution on accident because the surface/ volume ratio is huge.

The other way I've found to concentrate is the method of Farrell's lab. The Centricon-30s are pretty slow. I use the Centricon-100s and not much tubulin goes through. You have to presoak them in 1% triton then rinse them in H₂O just before use or they will bind all your tubulin. It's probably best to disregard fractions with low protein rather than dilute your product too much, as this method is pretty slow. Spin them at the max speed (1000xg). You have to stop them and resuspend the soln gently (especially down near the membrane) every 15 minutes or the flow will cease. This method takes several hours and you lose variable amounts of tubulin, typically around 30%. It helps a lot to re-use these concentrators. You lose a lot on the first use, but not so much on subsequent uses. Just store in 1% triton in cold and rinse again with dd-H₂O before use, however, don't allow the membrane to become dry once you wet it.

There is variability in how much tubulin you lose during concentration. Some of it seems to be due to polymer formation. For this reason, we wash out glycerol and GTP in the final steps so it's not present during concentration. Imidazole may also contribute so we dialyze into PEM buffer beforehand as well.

Yeast tubulin does not survive the freezer very well. When you thaw and clear it (20,000xg for 10') you may easily lose 30%. Keep that in mind when concentrating. However, the higher you try and raise the concentration, the more trouble you will run into. If you reach 1.5-2 mg/ml you are doing very well. Finally, drop freeze and store at -80C.

Yeast Tubulin Prep

- _____ Reserve 3 Beckman Centrifuges
- _____ Cool 3 Beckman Rotors & Tubes to 4C
- _____ Cool Sorval GSA Rotor & Tubes to 4C
- _____ Check out Microfluidizer

Solutions and Supplies (recommended amounts)

- _____ 1L cold H-PEM
- _____ 2L cold PEM
- _____ 2L cold PMG
- _____ 1L cold 25-PMG
- _____ 1.5ml 2M PMSF in DMSO
- _____ 5ml 1M DTT
- _____ 1ml 300mM GTP
- _____ 150ml Glycerol
- _____ 400ml Bed equilibrated DE52 (Whatman)
- _____ 300ml 5M NaCl
- _____ 5ml Bed 6xHis Resin (Ni-NTA from Qiagen)
- _____ 100ml 6xHis Binding Buffer
- _____ 100ml 6xHis Wash Buffer
- _____ 50ml 6xHis Elution Buffer
- _____ 50ml 500mM Imidazole

H-PEM :100mM PIPES, 2mM EGTA, 10mM MgSO₄, pH 6.9 w/NaOH

PEM :100mM PIPES, 1mM EGTA, 1mM MgSO₄, pH 6.9 w/NaOH

PMG :100mM PIPES, 1mM MgSO₄, 10% Glycerol, pH 6.9 w/NaOH

25-PMG :25mM PIPES, 1mM MgSO₄, 10% Glycerol, pH 6.9 w/NaOH

25-PM:25mM PIPES, 1mM MgSO₄, pH 6.9 w/NaOH

DE52 Bed Equilibrated in H-PEM + 160mM NaCl

6xHis Binding Buffer :25-PMG, 500mM NaCl, 20mM Imidazole, pH 7.4

6xHis Wash Buffer :25-PM, 20mM Imidazole, pH 7.4

6xHis Elution Buffer :25-PM, 350mM Imidazole, pH 6.9