

Microtubule Cosedimentation Assay Using *Drosophila* Extracts

(Adapted from Sisson, et al. (1997) Cell 90:235-245)

- 1) Homogenize dechorionated embryos in 2 volumes of C-buffer at 4°C. Use a motor-driven Teflon-tipped pestle and homogenize with 5 full strokes. Alternatively, homogenization can be performed with a glass dounce and 5 full strokes with a loose-fitting pestle followed by 5 full strokes with a tight-fitting pestle.
- 2) Let the homogenate sit at 4°C for 10 mins to promote microtubule depolymerization and then spin at 100,000 x g for 1 hour at 4°C.
- 3) The resulting supernatant (S100) is collected and placed on ice in a pre-chilled tube. Check the protein concentration with Bio-Rad Protein assay solution (Bio-Rad, Hercules, CA) using BSA as a standard. The concentration should be about 3-5 mg/ml.
- 4) Dispense equivalent aliquots of the S100 into four pre-chilled 1.5 ml microfuge tubes. These aliquots will be used to test the microtubule co-sedimentation properties of endogenous proteins under different conditions.
- 5) Supplement the S100 aliquots as indicated below at 4°C.
 - Tube #1: no supplement
 - Tube #2: supplement with taxol (Sigma) to 40 µM and GTP (Sigma) to 1 mM. Both help to stabilize microtubules.
 - Tube #3: supplement with taxol to 40 µM, GTP to 1 mM, apyrase (Sigma) to 80 U/ml, and AMP-PNP (Boehringer-Mannheim) to 0.5 mM. Apyrase is an ATP scavenger and AMP-PNP is a non-hydrolyzable ATP analog, both promote the binding of microtubule-based motor proteins to microtubules.
 - Tube #4: Same as tube #3.
- 6) Incubate each sample at 25°C for 20-30 mins and then layer each separately over a 10% sucrose cushion and centrifuge at 50,000 x g for 30 mins at 4°C. The cushions should be between 100-250 µl, depending on the volume of the sample. If the sample is 250-1000 µl a 250 µl cushion is recommended. The cushions for samples #2-#4 should be supplemented with taxol to 40 µM.
- 7) Supernatants (S50) are collected, frozen in liquid N₂ and stored at -80°C. Cushions are discarded. Pellets (P50) are washed with fresh C-buffer and samples #1-#3 are resuspended in C-buffer, while the sample #4 pellet is resuspended in CX-buffer. The volumes used to resuspend pellets should be the same as the initial S100 aliquot. Freeze the resuspended pellets from samples #1-#3 in liquid N₂ and store at -80°C.
- 8) The resuspended pellet from sample #4 is incubated on ice for 2-3 hours before layering it onto a 10% sucrose cushion (supplemented with taxol) and centrifuging as before. The resulting supernatant is frozen in liquid N₂ and stored at -80°C and the pellet is washed and resuspended in C-buffer, frozen and stored.

9) For SDS-PAGE, load 25-50 $\mu\text{g}/\text{lane}$ of each S50 and an equivalent proportion of each matched P50. For example, if you need to use 1/2 of an S50 to load 25 μg , than load 1/2 of the corresponding P50. You can assume that the concentration of the four initial S50's is the same as the S100; however, a Bradford assay should be performed on the S50 obtained from the extraction of the sample #4 pellet.

10) As controls protein blots should be probed with anti-tubulin (Sigma) and anti-KHC (DSHB, SUK4) antibodies.

C-Buffer

50 mM HEPES, pH 7.6
1 mM MgCl_2
1 mM EGTA
1:100 protease inhibitor cocktail
PMSF to 1 mM directly before use

CX-Buffer

C-buffer supplemented with the following:

10% glycerol
150 mM KCl
40 μM taxol
1 mM GTP
5 mM Mg-ATP

Stock Protease Inhibitor Cocktail

0.04 g Benzamidine HCl (10 μM)
0.003 g Phenanthroline (1.2 $\mu\text{g}/\text{ml}$)
0.025 g Aprotinin (10 $\mu\text{g}/\text{ml}$)
0.025 g Leupeptin (10 $\mu\text{g}/\text{ml}$)
0.025 g Pepstatin A (10 $\mu\text{g}/\text{ml}$)

Resuspend the indicated amounts of each of the protease inhibitors in 95% EtOH and store at -20°C . Dissolve the inhibitors in the EtOH at 37°C for a short time prior to use. Note that there will always be some undissolved material. Obtain inhibitors from Sigma. Final concentrations, after a 1:100 dilution in buffer, are indicated in parentheses above.