Fractionation of total RNPs/polysomes in sucrose gradients. (A. Barkan, 9/93, updated 2002)

If you use this method in published work, please cite A Barkan (1993) Nuclear mutants of maize with defects in chloroplast polysome assembly have altered chloroplast RNA metabolism. Plant Cell 5: 389-402.

KEEP EVERYTHING COLD. This does not have to be done in the cold room, but the sample should be kept on ice at all times, and buffers should all be cold. Also, keep in mind that sucrose gradients are quite unstable. Minimize agitation of the gradients. If using frozen tissue, be sure it does NOT THAW before it is fully ground and in extraction buffer.

1. Grind 0.2-0.4 gm tissue to powder in liquid nitrogen in mortar and pestle. (Do not use more tissue than this- using too much tissue can lead to incomplete inactivation of RNAses and degradation of the RNA).

Add 1 ml polysome extraction buffer and grind further, until thawed.
Force through glass wool plug in a 3 ml syringe to remove debris, collecting liquid into microfuge tube (on ice).
Let sit on ice for 10 minutes (to solubilize membranes).

2. Spin in microfuge for 5 minutes, in the cold, to pellet nuclei and junk. Remove the supernatant to a new tube.

3. Add 1/20th volume of 10% sodium deoxycholate.
Let sit on ice for 5 minutes (to complete solubilization of microsomal membranes).
Pellet remaining insolubles by microfuging for 15 minutes in the cold.

4. Load 0.5 ml of this mixture gently onto 4.4 ml analytical sucrose gradients (see below for gradient prep). Centrifuge in an SW50.1 rotor (or equivalent) at 45,000 rpm for 65 minutes at 4^{o}.

5. Prepare tubes for gradient fractions: label 12 microfuge tubes per gradient, and add to each tube 50 μl of 5%SDS/0.2M EDTA. (If you do not dissociate the protein from the RNA with SDS and EDTA before phenol extracting, the RNA will be drawn into the phenol phase). I routinely collect 0.41 ml fractions from the top of the gradient, with a pipetperson (slowly and carefully).

6. Add 0.4 ml phenol/chloroform/isoamyl alcohol (25:25:1), vortex, and microfuge to separate phases.

7. Add 1 ml of 100% ethanol at <u>room temperature.</u> Totally invert tube several times to mix sucrose with ethanol. If sucrose is not thoroughly mixed, RNA will not pellet and you will lose the RNA.

Microfuge at <u>room temperature</u> for 15 minutes to pellet the RNA. It is important to do this at room temperature to prevent the sucrose from pelleting in the lower gradient fractions. It is not necessary to add salt to these precipitations.

8. Pour off ethanol sup. Dry pellets briefly in SpeedVac, or let drain at room temp until almost dry. Resuspend in 30 μl cold TE. Store samples at -80^{o}. For Northerns, run 3 μl of each fraction on a gel. The rRNA bands should then be easy to see when the filter is stained with methylene blue.

CONTROLS

EDTA gradients: EDTA dissociates polysomes (and probably some other RNPs). To determine whether the sedimentation of the material of interest is sensitive to EDTA, add EDTA to 20 mM just before it is loaded onto the sucrose gradient. In addition, make the gradients with 10X EDTA salts rather than 10X polysome salts (same as polysome salts except that .01M EDTA is substituted for the $MgCl_2$.)

Puromycin release: This is a more rigourous test that rapid sedimentation is due to association with polysomes. In the presence of high salt, puromycin releases ribosomes from polysomes. I believe (but am not positive) that this results in 70S/80S ribosomes rather than in individual subunits. Release by puromycin into slowly sedimenting material is very good evidence that the material of interest is polysome associated.

After pelleting nuclei, but before adding the NaDOC, add KCl to 0.5M (it is already 0.2M so add an additional 0.3M). Add puromycin to 500 μg/ml. (Puromycin stock is 3 mg/ml in 1.5M KCl). Incubate at 37^o for 10 minutes. Add NaDOC to 0.5%, centrifuge for 15 minutes to pellet insolubles, and load onto sucrose gradient.

BUFFERS (Do not DEPC treat the sucrose stock if planning to use a UV detector, because this gives rise to a product that absorbs lots of UV).

		for 100ml
Extraction Buffer:	0.2M Tris-HCl, pH 9	20ml 1M
	0.2M KCl	1.5 gm
	35mM $MgCl_2$	0.71 gm
	25mM EGTA	0.95 gm
	0.2M sucrose	10 ml of 70%
	1% TritonX-100	1 ml
	2% polyoxyethylene-10-tridecyl ether	2 ml
	dd Water to 100 ml	

Filter through 0.2 μ sterile nitroclellulose unit, and store in aliquots in sterile, disposable plastic tubes (e.g. 50 ml conical) at -20^o.

Just before use add:

heparin to 0.5 mg/ml (from a 100 mg/ml stock)
B-ME to 100 mM (70 μl per 10 ml)
chloramphenicol to 100 μg/ml (from 50 mg/ml stock in ethanol)
cycloheximide to 25 μg/ml (from 10 mg/ml stock in water)

The antibiotics prevent run-off of chloroplast/mito ribosomes (chloramphenicol), and cytosolic ribosomes (cycloheximide).

10X polysome gradient salts:	0.4M Tris, pH 8.0
	0.2M KCl
	0.1M $MgCl_2$

Make RNAse free by dep treatment and autoclaving, or by filtering through 0.2 μ sterile filtration unit into RNAse-free container. Store at -20^o.

GRADIENT PREPARATION

The simplest method is to prepare many frozen step gradients at once, store them at -80°, and thaw them overnight at 4° before use.

Prepare stock solutions containing 15%, 30%, 40%, and 55% sucrose (as shown below). Pipet 1.1 ml of the 55% solution into the bottom of the 5 ml ultracentrifuge tubes, freeze at -80°, then pipet 1.1 ml of the 30% solution on top, freeze, etc.

For 25 4.4 ml gradients:

	15%	30%	40%	55%
70% sucrose (do not DEPC)	6.4 ml	13 ml	17 ml	24ml
10X polysome grad. salts	3 ml	3 ml	3 ml	3 ml
dep Water	20.5 ml	14 ml	10ml	3.3ml
100mg/ml heparin	150 µl	150µl	150µl	150µl
chloramphenicol (50mg/ml in etoh)	60 µl	60µl	60µl	60µl
cycloheximide (10mg/ml in water)	75µl	75µl	75µl	75µl

P.S. You can recover good quality total RNA from the remaining material that was not loaded onto the gradient. Simply add SDS to 0.5% and EDTA to 20 mM, phenol-chloroform extract and ethanol precipitate. Resuspend in TE, treat it with RNAse-free DNAse (BRL) for 10 minutes at 37° (20µg/ml), phenol-chloroform extract and ethanol precipitate.