Yeast Polyribosome Preparation - Proteins

References:

1. Ramirez, M., R. C. Wek, and A. G. Hinnebusch, 1991. Ribosome association of GCN2 protein kinase, a translational activator of the *GCN4* gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 11:3027-3036.

2. Deshmukh, M., Y.-F. Tsay, A. G. Paulovich, and J. L. Woolford, 1993. Yeast ribosomal protein L1 is required for the stability of newly syntesized 5S rRNA and the assembly of 60S ribosomal subunits. Mol. Cell. Biol. 13:2835-2845.

3. Atkin, A. L., N. Altamura, P. Leeds, and M. R. Culbertson, 1995. The majority of yeast UPF1 co-localizes with polyribosomes in the cytoplasm. Mol. Biol. Cell 6:611-625.

Schedule:

Day 1:
- Inoculate 5 ml yeast cultures, incubate overnight.
- Check solutions
- Pour sucrose gradients

Day 2:
- Inoculate 100 ml yeast cultures, incubate overnight.
- Chill rotors
- Thaw gradients
- Weigh out heparin and cycloheximide
- Label and prepare collection tubes + 1 control tube
- 250 ml centrifuge bottles in the refidgerator
- 45 ml screw capped centrifuge tubes

Day 3:
- Preparation
    - Turn on Spec 20
    - Turn on UV detector
    - Add the heparin, the cycloheximide and the DEPC to the lysis buffer
    - Ice
    - Chill centrifuges
- Harvest cells
- Prepare cell extract
- Load sucrose gradients
- Centrifuge
- Collect fractions

Day 4:
- Recover the proteins from the fractions

Day 5:
- Resolve the proteins by SDS-PAGE
- Transfer the proteins to nitrocellulose
- Begin immunoblotting

Day 6:
- Finsh immunoblotting

Day 7:
- Densitometry

Solutions:

Notes: Use RNase free stock solutions and labware. Follow the general guidelines in Sambrook et al., 1989 for the preparation of RNase free solutions and labware. Always add DEPC just before using the gradient buffers, the lysis buffer and the 60 % sucrose used for fraction collection.

Lysis Buffer

Require ~22.3 ml / sample

	For 100 ml
10 mM Tris HCl (pH 7.5, 4^{o}C)	1 ml 1M
0.1 M NaCl	2 ml 5M
30 mM $MgCl_2$	3 ml 1M
50 mg/ml cycloheximide	*5 mg (or 0.5 ml of a 10 mg/ml solution)
200 mg/ml heparin	*20 mg
0.2% DEPC	*2 ml 10% in EtOH

* Add just before use

60% Sucrose

120 g per 200 ml H_2O final volume.

Gradient Buffers:

	For 200 ml 47% Sucrose	For 200 ml 7% Sucrose
7% or 47% Sucrose	156.7 ml 60%	23.3 ml 60%
50 mM Tris Acetate (pH 7.0, 4^{o}C)	10 ml 1M	10 ml 1M
50mM NH_4Cl	10 ml 1M	10 ml 1M
12 mM $MgCl_2$	2.4 ml 1M	2.4 ml 1M
1 mM DTT	0.2 ml 1M	0.2 ml 1M
0.1% DEPC	2 ml of 10% in EtOH	2 ml of 10% in EtOH
	18.7 ml dH_2O	152.1 ml dH_2O

Preparation of 12 ml 7 - 47% Sucrose Gradients

1. Prepare the following sucrose solutions:

Final sucrose gradient buffer	Volume 7% sucrose gradient buffer per gradient	Volume 47 % sucrose gradient buffer per gradient
47 % sucrose	-	2.4 mls
37 % sucrose	0.6 mls	1.8 mls
27 % sucrose	1.2 mls	1.2 mls
17 % sucrose	1.8 mls	0.6 mls
7 % sucrose	2.4 mls	-

2. Pour the gradients:

Method 1:

1. Prepare 1-2 days in advance, store at 4°C.
2. Add 2.4 ml 7% sucrose to tube.
3. Underlay with 2.4 ml each of 17%, 27%, 37%, and 47% sucrose.

Method 2 (this is the method that I prefer):

1. Add 2.4 mls of 47% layer first and freeze at -70°C. Then add next layer and repeat until gradient is formed.
2. Store at 4°C for 24 hours to allow the gradients to thaw and diffusion to create a continuous gradient.

Chase solution

Add 100 ml of a 5 mg/ml solution of bromophenol blue to 49 ml of 60% sucrose.

POLYRIBOSOME ISOLATION:

All procedures are performed on ice unless otherwise stated.

CULTURE

1 Prepare 5 mls of yeast culture in YEPD or the appropriate medium to maintain selection for plasmids. Incubate with shaking at 30°C overnight.
2 Inoculate 100 ml YEPD or the appropriate medium. Inoculation of selective medium with 100 µl, 250 µl and 500 µl of the overnight culture the afternoon of the second day will generally ensure that at least one of the cultures will be at the correct OD_{600} the following day. Incubate overnight with shaking at 30°C.
3 Grow cells to an OD_{600} of 0.4 -0.6.

HARVEST

1. Pour culture into a 250 ml centrifuge bottle containing 5 mg Cycloheximide (500 µl of a 10 mg/ml cycloheximide solution; the final concentration of cycloheximide will be 50 µg/ml Cycloheximide) swirl for 30 sec and 100 mls ice.
2. Centrifuge in GSA rotor, 7 K RPM, 5 minutes.

CELLULAR EXTRACT PREPARATION

1. Resuspend cells in 20 ml cold lysis buffer. Transfer to 45 ml polyethylene tubes, and centrifuge in SS34 rotor, 7 K RPM, 3 minutes.
2. Suspend in 0.2 ml Lysis Buffer.
3. Transfer 0.4 ml to a 1.5 ml centrifuge tubes.
4. Add ice cold, baked acid washed glass beads to about 0.2 cm from the meniscus.
5. Vortex continuously for 3 minutes in a cold room.
6. Puncture a hole in the bottom of the tube with a hot 25 gauge needle and remove the cap. Place on top of a 1.5 ml centrifuge tube whose cap has been cut off. Place everything into a larger disposable tube and spin at 2 K RPM in a refridgerated centrifuge for 2 minutes.
7. Transfer supernatant to a 1.5 ml centrifuge tube.
8. Centrifuge at 9 K RPM (~6,500 g) for 10 minutes in an microcentrifuge in a cold room (or at 7 K RPM in the JA-18.1 rotor with the tube adaptors at a 45° angle in the Beckman high speed centrifuge).
9. Transfer supernatant to 1.5 ml sterile centrifuge tube.

Polyribosomes can now be collected by high speed centrifugation or analysed by density gradient centrifigation on sucrose gradients.

COLLECTION OF THE POLYSOMES BY HIGH SPEED CENTRIFUGATION:

1. Pellet polyribosomes by centrifugation at 160,000 g for 90 minutes.
2. Resuspend pellet in lysis buffer.
3. Store at -70°C.

ANALYSIS OF THE POLYRIBOSOMES BY DENSITY GRADIENT CENTRIFUGATION

LOADING SUCROSE GRADIENTS:

1. Dilute 5 µl of the cellular extract in 995 µl lysis buffer. Measure the OD_{260} and OD_{280}.
2. Apply the sample to the gradient with a pipettor. Place the tip of the pipettor at a 45°-60° angle to the tube wall approximately 2 to 3 mm above the gradient. Slowly dispense the sample and allow it to run down the side of the tube onto the gradient. The following amounts of sample provide the best resolution:

   20-50 ODU_{260} on a 35 ml gradient.

   10-20 ODU_{260} on a 12 ml gradient.

   5-10 ODU_{260} on a 5 ml gradient.

   Note: 1 0DU = 1 ml of solution at a concentration that gives an OD reading of 1. Therefore ml loaded = ODU Loaded x 1000 ml x $(OD_{260})^{-1}$.
3. Add 1/24 of the volume of sample loaded on the gradient to the control tube. Store on ice.

DENSITY GRADIENT CENTRIFUGATION:

Centrifuge the sucrose gradients in a swinging bucket rotor at 4°C at the appropriate speed and time:

27K RPM, 4 hours in SW26 (35 ml gradient)

39 K RPM, 2.5 hours in SW41 (12 ml gradient)

47K RPM, 40 minutes in SW 50.1 (5 ml gradient)

Rotors can be accelerated rapidly without disturbing the gradient/sample interface and braked without disturbing the separation.

Note: This is a Rate Zonal Technique

COLLECT FRACTIONS:

Equipment required:

- Pump (preferably an infusion-withdrawal syringe pump)
- UV detector equiped with a density gradient flow cell
- Chart recorder
- Fraction collector

I use an Isco density gradient fractionator (Model 640) equiped with a 5 mm pathlength density gradient flow cell with an aperture of 1 mm, and a UA-6 UV/Vis detector with built in chart recorder. Isco no longer makes the density gradient fractionator. Similar results can be obtained by an Isco ProTeam LC™ system (UA-6 UV/Vis detector and recorder, Tris pump, Foxy Jr fraction collector) equiped with a 5 mm pathlenght density gradient flow cell.

Set Up:

1. Insert the correct size collar and ring, and adjust the scale height setting for the centrifuge tube size.
2. Adjust the settings on the detector:
   - Sensitivity - SET LAMP AND OPTICS
   - Noise filter - 1.5

- Peak separator - off
- Chart speed - 30

3. Fill the syringe with chase solution.
4. Fill a centrifuge tube with 7 % sucrose gradient buffer. Remove the retaining nut on the bottom of the flow cell. Slip the retaining nut, ring, and rubber collar over the end of the centrifuge tube. About 1 mm of the top of the centrifuge tube should be exposed. Insert the tube into the flow cell. Tighten the retaining nut. The nut should only be snugged up. Check to ensure the centerline of the tube is vertical. Straighten if necessary by gently pushing the tube into position.
5. Pierce the bottom of the tube.
6. Select the flow rate - 1.0.
7. Zero the syringe displacement counter with the reset pushbutton.
8. Turn the syringe direction switch to the FWD position.
9. When the flow cell is filled with the 7 % sucrose gradient buffer, the baseline can be adjusted.
    - Set the UA-6 detector to SET LAMP AND OPTICS.
    - Set the BASELINE ADJUST control to MAX. open.
    - Adjust the BASELINE ADJUST control of the optical unit until the chart pen moves to near zero. (If the indication from the previous step cannot be obtained, turn the BASELINE ADJUST control of the optical unit to maximum open. Move sensitivity switch off of SET LAMP AND OPTICS to desired absorbance range.)
    - Set the desired sensitivity (Usually 0.5 to 1.0. 1.0 works best for a 12 ml gradient loaded with 10 ODU).
    - Align RECORDER OFFSET on the UA-6 control to it's mark.
    - Activate AUTO BASELINE. The pen should deflect near midscale. Adjust the baseline setting to the 20 mark.
10. Once a stable baseline has been established, turn the syringe direction switch to the REV position. Set the flow rate at a maximun of 5.0. Collect the chase solution. When the top of the chase solution is just above the tip of the needle
    - Turn the syringe direction to OFF.
    - Remove the centrifuge tube.
    - Lift the pen.
    - Clean the flow cell, collar and ring.

Collecting the samples:

11. Install the first sample tube as described in step 5.
12. Pierce the bottom of the tube.
13. Select:
    - Collection size - 0.6 mls
    - Flow rate - 1.0
    - Put the pend down on the chart paper.
    - Put the collection tubes into the collector and align the discharge spout over the first collection tube.
14. Turn the syringe direction switch to the FWD position.
15. When the first drop appears at the discharge spout, turn the syringe direction OFF.

16. Zero the syringe displacement counter with the reset pushbutton and set the collection mode on VOLUME.
17. Turn the syringe direction switch to the FWD position..
18. Collect sample until the blue chase solution appears at the discharge spout.
19. Turn the syringe direction switch to the REV position to collect the chase solution. Leave the fraction collector on until it changes tubes. When the fraction collector changes tubes, immediately turn the collection mode OFF. Remove the collection tubes. They can be stored at -20°C until the RNA can be extracted. Remove the centrifuge tube.
20. Wash the flow cell, collar and ring.
21. Repeat steps 11 to 20 for each sample to be collected.

Clean up:

22. After all the samples have been collected, clean the system with 50 mls of warm water containing 2 drops of dish detergent. Rinse with 250 mls of distilled water. This step is important to prevent the growth of fungus within the flow cell and the tubing.

PURIFICATION OF THE PROTEINS FROM THE DENSITY GRADIENT FRACTIONS

Solutions:

- 7:2 Acetone: dH_2O, stored at -20°C
- 1 X Protein sample buffer

Method:

1. Add 900 µl of a 7:2 Acetone: dH_2O. Mix by vortexing each tube for 10 seconds.
2. Incubate at -20°C for at least 30 minutes.
3. Pellet the proteins by centrifugation in a microcentrifuge for 5 minutes at full speed.
4. Pour the supernatant off*. Asperate to remove all traces of acetone.
5. Air dry the pellet by placing the tubes into a 37°C incubator for 5 minutes.
6. Resuspend the pellet in 10 µl of 1 X protein sample buffer.
7. The proteins recovered from the sucrose gradients are now ready to be analyzed by Western blotting.

Note: *Acetone is a P list chemical. It must be collected into the used acetone container.

The relative distribution of 80S ribosomal particles to polyribosomes on the sucrose gradients can be altered by the following methods:

1. Ribosome Run Off:
   - Chill culture on ice for 5 minutes before harvest.
   - Prepare extracts in the absence of cycloheximide.

2. Disruption of polyribosomes by RNase Treatment:
   1. Prepare cell extract in the absence of DEPC.
   2. Add 5 µl RNase A (10 mg/ml) to 200 µl cell extract. Incubate 15 min on ice immediately before loading the sucrose gradients.

3. Altered growth conditions:
   Shift the cells from a synthetic medium to YEPD for two generations before harvesting the cells.
   1. Grow cells in 100 mls of synthetic medium to an OD_{600} of 0.1 to 0.15. Pellet the cells and resuspend them in 100 mls of YEPD.
   2. Grow with shaking to an OD_{600} of 0.4 to 0.6.