

Yeast Polyribosome Preparation - RNA

References:

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2. 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.
3. Baim, S. B., D. F. Pietras, D. C. Eustice, and F. Sherman, 1985. A mutation allowing a mRNA secondary structure diminishes translation of *Saccharomyces cerevisiae* Iso-1-Cytochrome *c*. *Mol. Cell. Biol.* 5:1839-1846.
4. Leeds, P. S. W. Peltz, A. Jacobson, and M. R. Culbertson, 1991. The product of the yeast *UPF1* gene is required for rapid turnover of mRNAs containing a premature translational termination codon. *Genes and Dev.* 5:2303-2314.

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 refrigerator
- 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:

- Extract the RNA from the fractions

Day 5:

- Resolve the RNA on formaldehyde-agarose gels
- Set up Northern transfer

Day 6:

- Set up hybridization

Day 7:

- Finish hybridization
- Phosphorimaging and autoradiography

Solutions:

Notes: Use RNase free stock solutions and labware. Follow the general guidelines in Sambrook et al. 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

	<u>For 100 ml</u>
10 mM Tris HCl (pH 7.5, 4°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 H₂O final volume.

Gradient Buffers:

	<u>For 200 ml</u> <u>50% Sucrose</u>	<u>For 200 ml</u> <u>15% Sucrose</u>
15% or 50% Sucrose	166.7 ml 60%	50 ml 60%
50 mM Tris Acetate (pH 7.0, 4°C)	10 ml 1M	10 ml 1M
50mM NH ₄ Cl	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 ₂ O	152.1 ml dH ₂ O

Preparation of 12 ml 15-50 % Sucrose Gradients

1. Prepare the following sucrose solutions:

<u>Final sucrose</u> <u>gradient buffer</u>	<u>Volume 15 % sucrose</u> <u>gradient buffer per gradient</u>	<u>Volume 50 % sucrose</u> <u>gradient buffer per gradient</u>
50 % sucrose	-	2.4 mls
41.25 % sucrose	0.6 mls	1.8 mls
32.5 % sucrose	1.2 mls	1.2 mls
23.75 % sucrose	1.8 mls	0.6 mls
15 % 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 15 % sucrose to tube.
3. Underlay 2.4 ml each of 23.75 %, 32.5 %, 41.25 %, and 50 % sucrose.

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

1. Add 2.4 mls of the 50 % 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 μ l of a 5 mg/ml solution of bromophenol blue to 50 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 OD₆₀₀ the following day. Incubate overnight with shaking at 30°C.
3. Grow cells to an OD₆₀₀ of 0.4 -0.6.

HARVEST

1. Pour culture into a 250 ml centrifuge bottle containing 5 mg (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 at full speed 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 refrigerated 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 a 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.

ANALYSIS OF THE POLYRIBOSOMES BY DENSITY GRADIENT CENTRIFUGATION

LOADING SUCROSE GRADIENTS:

1. Dilute 5 μ l of the cell extract into 995 μ l of the lysis buffer. Measure the OD₂₆₀ and OD₂₈₀.
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 ODU₂₆₀ on a 35 ml gradient.
 - 10-20 ODU₂₆₀ on a 12 ml gradient.
 - 5-10 ODU₂₆₀ on a 5 ml gradient.

Note: 1 ODU = 1 ml of solution at a concentration that gives an OD reading of 1. Therefore μ l loaded = ODU Loaded x 1000 μ l x (OD₂₆₀)⁻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 equipped with a density gradient flow cell
- Chart recorder
- Fraction collector

I use an Isco density gradient fractionator (Model 640) equipped 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) equipped with a 5 mm pathlength 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 15 % 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 15 % 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 maximum 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 pen 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 RNA FROM THE DENSITY GRADIENT FRACTIONS

Solutions:

Tris buffered phenol
Chloroform
8M LiCl
Absolute ethanol
70 % ethanol

Method:

1. Extract the control and the fractions with phenol:chloroform as follows:
 - Add 600 μ l of 1:1 Tris buffered phenol:chloroform to each fraction and the control tubes.
 - Vortex the samples for 20 seconds.
 - Separate the aqueous phase from the organic phase by centrifuging the tubes in a microcentrifuge for 2-3 minutes at full speed.
2. Remove the aqueous layer to a new microcentrifuge tube. Repeat the phenol:chloroform extraction.
3. Remove 450 μ l of the aqueous layer to a new microcentrifuge tube.
4. Ethanol precipitate the RNA as follows:
 - Add 50 μ l 8M LiCl and 1.2 mls of absolute ethanol.
 - Incubate for one hour at -20°C .
 - Pellet the RNA by centrifugation at full speed in a microcentrifuge for 10 minutes to pellet the RNA.
 - Asparate the supernatant being careful not to disturb the pellet.
5. Wash the RNA pellet.
 - Add 1 ml of 70 % ethanol.
 - Vortex at full speed for 20 seconds.
 - Centrifuge at full speed for 5 minutes.
 - Remove the supernatant by aspiration.
 - Incubate the open tube at 37°C for 5 minutes to dry the pellet.
6. Dissolve the RNA pellet in 11.25 μ l dH_2O . Heat to 65°C for 10 minutes to assist with resuspension. Vortex. Centrifuge briefly.

Note: Use the entire fraction for Northern analysis.