

Ribosome Fractionation in Yeast

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[Abstract] This protocol describes yeast ribosome fractionation in the gradient of sucrose. During the cyclic process of translation, a small (40S) and large (60S) ribosomal subunit associate with mRNA to form an 80S complex (monosome). This ribosome moves along the mRNA during translational elongation, and then dissociates into the 40S and 60S subunits on termination. During elongation by one ribosome, further ribosomes can initiate translation on the same mRNA to form polysomes. The mass of each polysomal complex is determined primarily by the number of ribosomes it contains. Hence, the population of polysomes within the cell can be size-fractionated by sucrose density gradient centrifugation on the basis of the loading of ribosomes on the mRNA. Several compounds help to maintain or to disrupt the polysomes (Figure 1).

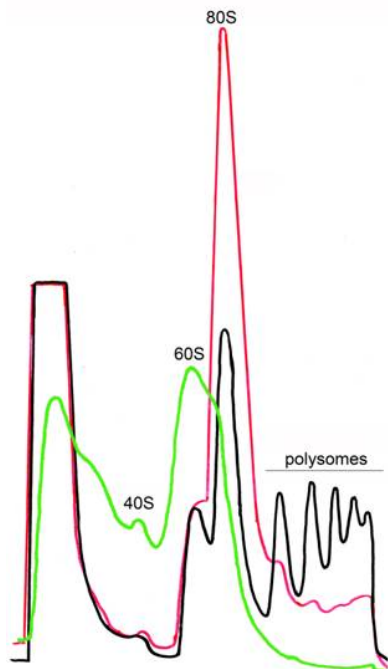


Figure 1. Ribosome profiles after sucrose gradient centrifugation. Ribosome fractionation was performed in the presence of CHX (black), in the absence of CHX (red) or in the presence of EDTA (green). CHX stabilizes polysomes. In the absence of CHX polysomes are destroyed but 80S is still present. 80S can be completely disrupted in cell extracts by treatment with EDTA.

Materials and Reagents

1. Glass beads for cells breaking 0.5 mm (Bio Spec Products, catalog number: 110/9105)
2. Cycloheximide (CHX) (Sigma-Aldrich, catalog number: C7698) solution 100 mg/ml prepared on ethanol
3. Bradford reactive (Bio-Rad Laboratories, catalog number: 500-0006)
4. Protease inhibitor cocktail (F. Hoffmann-La Roche, catalog number: 13560400)
5. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: P7625) 100 mM solution prepared on isopropanol
6. 100% trichloroacetic acid (TCA) (Fluka, catalog number: 91230)
7. Tubes for SW41 rotor Ti (Beckman Coulter, catalog number: 331372)
8. Protease inhibitor cocktail (F. Hoffmann-La Roche, catalog number: 1836170)
9. Lysis buffer
10. Ethanol
11. Laemmli sample buffer
12. Bromophenol blue
13. YPD media (see Recipes)
14. Buffer A (see Recipes)
15. 2x buffer B2 times concentrated (see Recipes)
16. Sucrose gradient (see Recipes)
17. Foni-inert (see Recipes)

Equipment

1. Table Centrifuges
2. Glass bead beater Genie Disruptor (Scientific Industries, catalog number: SI-DD38)
3. Ultracentrifuge
4. Rotor SW41 Ti (Beckman Coulter, catalog number: 331362)
5. Density Gradient Fractionation System (ISCO, catalog number: 67-9000-177)

Procedure

1. Yeast cultures were grown on YPD or selective media. In the morning dilute night culture till $OD_{600} = 0.15$. Grow 100 ml of culture at 30 °C until $OD_{600} = 0.6-0.8$.
2. To keep the polysomes add cycloheximide (CHX) till final concentration 0.1 mg/ml and incubate 10 min on ice.

3. Spin the cells for 5 min at 4,000 x g and wash with 50 ml of cold water containing 0.1 mg/ml of CHX.
4. Resuspend the pellets in 1 ml of lysis buffer containing 0.1 mg/ml of CHX, spin. Cells can be frozen and stored at -20 °C.
5. Work on ice! Resuspend the pellet in 0.4 ml of buffer A, add 0.5 ml of glass beads and disrupt 15 min on the glass bead beater at 4 °C.
6. To avoid the contamination with cells debris preclean the total extracts. For this transfer the liquid phase into the new tube. Centrifuge 1 min 16,000 x g at 4 °C.
7. Transfer the supernatant into the new Eppendorf tube and centrifuge 10 min 16,000 x g at 4 °C.
8. Measure the total protein concentrations in the supernatants [= TE (Total Extracts)]. For this dilute the total extracts 1:20 and load 5 and 10 µl to the Bradford sample (0.8 ml of water and 0.2 ml of Bradford reactive).

Note: Lysate should be fresh, they can not be frozen!

9. Pour 12 ml gradients (7 to 47% sucrose) using Density Gradient Fractionation System (ISCO) (Figure 2). Gradients are best poured at the night before the centrifugation. Keep the gradients at 4 °C before loading samples. Balance gradient volumes in paired rotor buckets firstly by eye, carefully removing some of top of gradient using a pipette tip if necessary. Check with the balance.

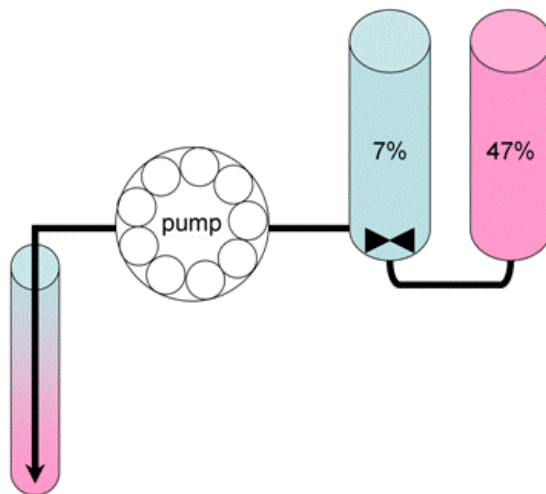


Figure 2. Preparation of sucrose density gradients for polysome analysis. 6 ml of 7% (marked in blue) and 6 ml of 47% (marked in pink) sucrose solutions are placed into the front and back compartments of a gradient pourer, respectively. A small magnetic flea is used to mix the solution in the front compartment, which is drained slowly using a peristaltic pump to the bottom of the centrifuge tube (approx 10 min per gradient). Care should be taken to minimize the mixing in the centrifuge tube.

10. Load 0.2 ml of the total extracts containing 2-4 mg of total protein on the top of 7-47% sucrose gradient without disturbing the surface.

Note: Do not load more than 0.25 ml of the total extracts!

11. Centrifuge for 150 min at 220,000 x g (39,000 rpm for rotor SW41) at 4 °C.
12. Before spin is complete, set up to analyze gradient(s). Standardize absorbance detector with a 1x buffer B. Set baseline according to the Instruction Manual.

Note: Before putting first gradient on make sure the pump line is filled with foni-inert so that no bubbles can enter sample - this will destroy the gradient! Try briefly raising the needle and running 1 ml of foni-inert into the base cup just prior to first fractionation.

13. Carefully remove gradients and store at 4 °C without disturbing. Gradients must be fractionated before they start to diffuse. Insert first gradient snugly into top of fractionator [Density Gradient Fractionation System (ISCO)] and raise lower platform to base of tube, and screw-lock. Slowly screw up needle into bottom of gradient tube (Figure 3). Run the pump.

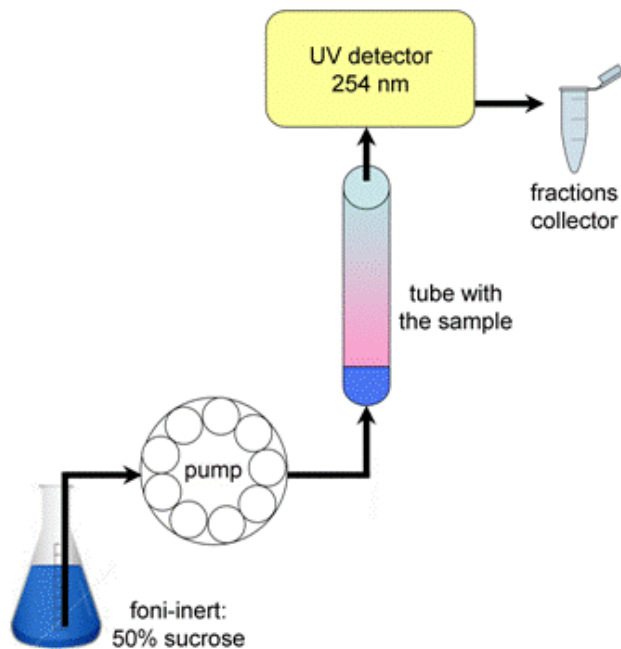


Figure 3. Fractionation of polysome gradients. Pierce the tube with the needle. Using a peristaltic pump, extrude the gradient from the tube with a foni-inert through a spectrophotometer that monitors the absorbance at 254 nm, and then fractionated into Eppendorf tubes.

14. End run when first droplets of foni-inert are collected at end of gradient, then put the pump in the reverse mode and pump back most of the foni-inert for reuse. Be careful that no new air bubbles are introduced into pump line and needle during this procedure.
15. Clean up after fractionating all parts of the gradient fractionation system. Rinse entire system with water, then with 20% ethanol. Clean up bench and equipment. Discard waste solutions and trash.

Density Gradient Fractionation System (ISCO)

Isco Type 6 Optical Unit:

Use 254 nm filter and source screen

UA-5 Absorbance Detector Settings:

Chart Speed: 30-60 cm/h

Sensitivity: 1.0 or 2.0 (A_{260} Units = Full scale)

Slope Sensitivity = High

Pump: Setting 40, clockwise

Fraction Collector: (if desired)

Use 1.5 ml microfuge tubes inside larger glass tubes

Collect 0.4 min drops (0.75 ml)

16. Take 0.4 ml of fraction (the rest freeze) and add 60 μ l of 100% TCA, incubate for 10 min on ice.
17. Spin at 16,000 $\times g$ for 15 min at 4 °C.
18. Aspirate all traces of TCA and resuspend the pellets in 50 μ l of Laemmli sample buffer.
19. Load 10 μ l on SDS-PAGE

Note: In case of polysomes disruption, polysomes were dissociated by treatment with 25 mM EDTA added instead of CHX, in the buffer A and in the gradient.

Recipes

1. YPD media
 - 2% glucose
 - 2% bactopectone
 - 1% yeast extract
2. Buffer A
 - 20 mM Hepes (pH 8.0)
 - 50 mM KCl
 - 10 mM $MgCl_2$
 - 1 % Triton X-100
 - 1 mM DTT

- 1 mM PMSF
- CHX 0.1 µg/ml
- Protease inhibitor cocktail 1 tablet for 10 ml of extract
- 3. 2x buffer B2 times concentrated
 - 40 mM Hepes
 - 100 mM KCl
 - 20 mM MgCl₂
- 4. Sucrose gradient
 - 7% sucrose on 1x buffer B + 0.1 mg/ml CHX
 - 47% sucrose on 1x buffer B + 0.1 mg/ml CHX
- 5. Foni-inert
 - 50% sucrose on water
 - 0.01% bromophenol blue

Acknowledgments

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2. Panasenko, O. O. and Collart, M. A. (2012). [Presence of Not5 and ubiquitinated Rps7A in polysome fractions depends upon the Not4 E3ligase](#). *Mol Microbiol* 83(3): 640-653.