Yeast Lysis and Polysome profiling by Sucrose gradient

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This protocol is for polysome fractionation by sucrose gradient, to view translational status of cells and ribosome-association of mRNAs and proteins, using the BioComp gradient station (http://www.biocompinstruments.com/). It's based on the protocols from David Weinberg's lab at UCSF, and BioComp instructions.

Components

Wet ingredients

- Sample: Yeast in desired conditions, about 50mL liquid media, about OD 0.4-0.8.
- Yeast Polysome gradient buffer: 120mM KCl, 7.5mM MgCl2, 20mM HEPES-KOH pH7.5 (NOTE: Can adjust salt, Mg, pH, etc; common additions are cycloheximide to freeze ribosomes in place, heparin to reduce nonspecific RNA-protein binding and inhibit RNase, DTT as reducing agent, RNase inhibitors. I've made good polysome gradients without any of these at 2-10mM MgCl2, pH 6.5-7.5, with Tris or HEPES. Degraded DTT is bad as it absorbs over the RNA spectrum. This protocol chooses to have heparin, cycloheximide, RNase-In in the lysis buffer, not the gradient buffer.)
- Yeast Lysis buffer (YLB): 120mM KCl, 7.5mM MgCl2, 20mM HEPES-KOH pH7.5, 1% Triton X-100, 2mM DTT, 100 ug/mL cycloheximide, 20 U/mL SUPERase-In, 1X cOmplete EDTA-free Protease Inhibitor Cocktail, 200 ug/mL heparin (Sigma). Make large stock of KCl, MgCl2, HEPES, Triton. To 50mL gradient buffer stock, on ice, add just before use:

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  - 1 tablet of Protease inhibitor
  - 100uL of cycloheximide stock (50mg/mL in DMSO)
  - 50uL of SuperAse-In stock (20U/uL)
  - 1mL Heparin stock (10mg/mL)
  - 100uL of DTT stock (1M)
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- 50% Sucrose buffer: 0.5g/ml sucrose in polysome gradient buffer, filter sterilized.
- 10% Sucrose buffer: 0.1g/ml sucrose in polysome gradient buffer, filter sterilized.

Dry ingredients

- BioComp Instruments gradient station.
- Beckman Coulter S-rated ultracentrifuge, e.g. Optima XP-100.
- Beckman SW40Ti rotor and SW40Ti tube buckets, kept in cold room. Rotor must be placed only on rotor-holding platform, do not scratch speed ring on bottom.
- Seton open-top polyclear centrifuge tubes SW28.1 (part.no 7042).

Lyse cells

CAUTION: Wear safety goggles for this, certainly until lysate is thawed (step 6). Liquid nitrogen, steel balls, mixer mills, and frozen plastic, can all cause things to fly around and damage you, especially your eyes. No explosions: avoid sealing a container with liquid nitrogen in it.

1. Filter 50mL of cells on a Whatman 0.5um nitrocellulose filter under vacuum. Just before the filter starts to dry, disassemble filter apparatus and throw the filter into a 50mL tube in liquid nitrogen (LN; that you previously prepared).

Pour off excess LN from the 50mL tube and allow remaining to bubble off in -80 for 15 mins (!No explosions!). Then cap the tube and keep in -80C until ready for next step.

2. Thaw 50mL tube with filter 1 min on ice. Wash cells off filter with ~1mL ice-cold freshly made YLB. Spin at 4C and remove supernatant. With a wide-mouth tip, resuspend in 100uL YLB and re-freeze in balls on the side of 2mL safe-lok tubes in LN, about 200uL material. Uncap tubes in -80C for 5min to allow LN to bubble off (!Still no explosions!).
3. Add pre-chilled 7mm steel balls to the safe-lok tubes. Cap tubes. Place in to pre- chilled vials for mixer mill, Retsch MM400. Chill filled vial (place in LN until boiling stops).
4. Grind on mixer mill, 4 cycles of 90s x 30Hz. Chill vials in LN after each cycle. You can leave tubes in -80 here if needed, which also speeds the thawing in next step.
5. Remove tubes from vials and place on ice. To each tube add 500uL of ice-cold YLB. Cap tube and mix gently by inversion until sample is thawed. Remove steel ball with magnet, carefully avoiding sample loss.
6. Remove 100uL of the lysate (true Total), and freeze in a 1.5mL tube.
7. Clarify spin, 3,000g x 30sec, 4C, to remove debris and unlysed cells. Save pellet for troubleshooting and take supernatant into fresh 1.5mL tube (on ice). Record OD on nanodrop, blanking against YLB, if possible.
8. Spin out RNA-protein granules and membranes: 20,000g x 10min, 4C. Take supernatant to fresh 1.5mL tube; this will be put on sucrose gradient. Freeze the pellet (P20), containing RNA-protein granules and some membrane-associated components. You can extract RNA and/or proteins from Total P20 while sucrose gradient is running.

Prepare gradients

1. Layer sucrose into centrifuge tube: place a polyclear centrifuge tube in the marker block. Pour 10% sucrose buffer into the tube up to the top of the marker block (roughly 10ml for SW28.1 / 19.5ml for SW28). Load 50% sucrose buffer into a 50ml Luer-lok syringe with a layering cannula (BioComp 106-211) attached; with the needle pointing upwards, expel air from the needle. Quickly and smoothly invert the syringe so the needle is in the bottom centre of the half-filled centrifuge tube. Slowly layer 50% sucrose buffer at the bottom of the tube until the top of the meniscus is at the top of the tube (roughly 10ml; 19ml for SW28), and carefully remove needle from tube. Place the capillary cap on top of the tube, ensuring tube is sealed round edges of cap. Repeat for desired number of tubes (2, 4 or 6 total), and place filled tubes in magnetic tube rack.
2. Level the gradient maker platform on the gradient station: turn gradient-maker on, choose GMST menu option. The machine will prompt you to level the gradient-making platform. Place a bubble level on the platform with axis perpendicular to the side plate of the machine and use the UP and DOWN menu options to level the platform (the machine should already be level front to back); press DONE.
3. Make gradients: place magnetic tube rack on gradient-making platform. Select GRAD option to arrive at gradient menu. The first time, go to LIST and choose the SW40Ti rotor option. Press DOWN until arriving at the 10-50% sucrose gradient option, and press USE. If the machine was used for 10-50% sucrose gradient immediately previously, simply select LAST from the gradient menu. Press USE to start making gradients, which takes roughly 10 minutes. *From this point onwards, keep the tubes upright and make no sudden movements with them, so as not to disturb the gradient.* Remove capillary cap from tube and, using long-nosed pliers if necessary, place in rotor bucket in bucket rack.
4. Balance the tubes: balance tube 1 with tube 4, 2 with 5, and 3 with 6. Placing bucket-tube assembly on scale, remove sucrose gradient from top of tube as necessary to ensure paired tubes are within 0.1g in mass.
5. Prepared gradients can be stored at 4C for a day or two.

Spin

6. Load sample and assemble rotor: take sample, SW40Ti rotor, bucket rack, and 200 ul pipette to ultracentrifuge. Load sample in each tube by placing filled pipette tip in meniscus at side of tube, and pipetting slowly; you should see the sample spreading out across top of liquid. Gently place lid on tube and screw cap on bucket. Hang buckets in numbered slots on rotor, checking that both hooks are attached for every bucket.
7. Set up centrifuge: turn on centrifuge, break vacuum and open spin chamber. Select !!!27500 rpm, 2hrs, 4C, with vacuum, on centrifuge controls. Place rotor assembly on axle, and seal spin chamber. Start centrifuge and fill out centrifuge logbook. Check on the centrifuge after 15 minutes to ensure it is running smoothly.

8. After running for 2hrs, the centrifuge takes several minutes to brake. Once the centrifuge has stopped spinning, release the vacuum, carefully remove rotor from spin chamber and place buckets in bucket rack.

Set up gradient station

9. Take bucket rack with gradients to gradient station, very gently.
10. Turn on the gradient station, the UV monitor, the fraction collector, and the linked computer. Connect the USB cable, start the gradient profiler program on the computer and enter appropriate parameters.
11. Flush the line and calibrate the UV monitor: press DRAIN on fraction collector. On the gradient station, from the initial screen press FRAC, then FRAC. Hold RINSE on gradient station to flush the line. Half-fill a centrifuge tube with 10% sucrose buffer, turn front dial so that vacuum plunger descends at about 0.5 mm/s. Once plunger is a few cm into liquid and drips are coming out of the fraction collector, press AUTO ZERO on the UV monitor.

Collect fractions

For each ultracentrifuge tube with sample:

12. Initialize fraction collector: ensure there are 30x clean 1.5ml tubes in the two middle rows of the tube holder. Pre-label the tubes if desired. Press END, then START, and make sure drip outlet is above tube 0.
13. Remove bucket cap, remove centrifuge from bucket using long-nosed pliers, and attach locking top to centrifuge tube. Place tube in tube holder on gradient station, locking the tube in place by rotating the cap to lock in place. Slide tube holder onto mount on top of gradient station with window facing to the right, and turn clockwise so window is facing towards you.
14. On gradient station, press FRAC once or twice to get to the fractionation menu, then SNGL for single run, and set the parameters to speed = 0.3, distance = 3.2 (for SW28.1; 2.6 for SW28), and 31 fractions. Rotate front dial to full counterclockwise position. Move plunger downwards by turning dial to the right until speed = 1.0 mm/s; move plunger slowly (0.2 mm/s) as it approaches the gradient surface, so that you can stop (by turning dial fully left) as soon as the plunger has sealed on the gradient. Press RESET on gradient station. In subsequent tubes from same run, reset to same position – i.e. 0.0 mm on display – from previous tube.
15. On the gradient profiler program on the computer, press RECORD. Then press START on gradient station.
16. When finished, remove tubes from fraction collector and label them. Press EXIT 3 times on gradient station to return to fraction menu, and remove centrifuge tube from holder. *Crucially, save the output on the computer, and press NEW RUN to record the next gradient.*
17. Note that the first 1-3 tubes in the fraction collector are usually empty, so that the tube number on the rack is offset from that reported in the gradient profiler software (BioComp instructions suggest this is solvable: good luck!). Tube 0 according to gradient profiler software is the first filled tube on the fraction collector. It may be easiest to label the tubes accordingly, after the fractions are collected.

Cleanup

18. Check **all** equipment for potential sucrose gradient spills and clean thoroughly with damp cloth and dilute ethanol; this is a very sticky spill. In particular, clean the plunger and flush the tubing with water.
19. Flush tubing with a full tube of 50% Ethanol to disinfect (DRAIN on fraction collector, from FRAC screen on gradient station lower plunger at about 0.5 mm/s).
20. Store rotor and buckets in cold room, on rotor holding platform.