RNA content of RNP granules in yeast

Edward Wallace, ewjwallace@gmail.com

April 15, 2015

Introduction

The aim is to find out which RNAs are sequestered in RNA-protein assemblies, by extracting both supernatant and pellet of 100,000g fractionated lysate.

Prepare

- soluble RNA buffer (SRB; 20mM HEPES-NaOH pH7.4, 120mM KCl, 2mM EDTA, 0.2mM DTT, 1:100 Protease Inhibitors cocktail IV, RNAsin plus.) Make stock of salt and buffer, then chill and add DTT and inhibitors immediately before use.
- insoluble RNA buffer (IRB; 20mM HEPES-NaOH pH7.4, 120mM NaCl, 5mM EDTA, 2% SDS, 2mM DTT, 1:100 Protease Inhibitors cocktail IV, RNAsin plus.).
- Prepare appropriate numbers of safe-lok tubes loaded with 7mm steel balls, racked in LN, labeled on sides and top. Also, labeled tubes for all steps of protocol.
- Waterbath at desired temperature, ice, chilled centrifuge rotors, etc.

CAUTION: Phenol and Phenol:Chloroform are extremely dangerous, causing chemical burns, and must be handled in a fume hood.

Sample growth and lysis

1. Grow up several cultures of yeast (BY4741) to 4×10^6 cells/mL ($OD_{600} \approx 0.4$) at 30°C, in 100ml media in a 250ml flask.
2. Transfer 2×10^8 cells to a 50mL conical tube (50 mL of a 4×10^6 cells/mL culture).
3. Spin at 2500g for 30s. Gently decant and discard supernatant.
4. For short heat shock treatment, hold tube containing pellet in waterbath at desired temperature for desired time.
5. Resuspend pellet in 100 μl ice-cold SPB, on ice.
6. Drip resuspended pellet onto upper wall of tube containing steel ball. Goal is to get a nugget of frozen material on the wall, and to avoid dripping the material around the ball and thus freezing the ball to the bottom of the tube; having some LN2 remaining in the tube helps.
7. Place tubes at -80°C; when all LN2 has boiled out of tube (listen – if any popping or hissing, keep waiting), snap tube closed carefully, away from other tubes. Keep in LN2. (Any remaining LN2 in tube will cause tube to explode open and fire the stainless steel ball into your iPad, brain, colleague, or other important equipment.)
8. Rack the tube into the PTFE 2mL tube adaptor for the Retsch Mixer Mill MM400 (Retsch #22.008.0005) and submerge the entire assembly in LN2. Agitate for 4×90 seconds at 30 Hz in a Retsch Mixer Mill MM400, returning sample holder to LN2 between sessions. Complete lysis produces fine snowy powder in the tube.
9. Remove sample tubes from LN2 and pop the caps to relieve pressure.
10. Add 400 μl SPB to each tube, and extract ball with a magnet. We rinse balls in methanol and store in 50% ethanol.

Soluble fraction extraction

11. Spin at 3000g for 30 seconds (clarification step) to remove whole cells and very large aggregates.
12. Decant clarified liquid into a 1.5mL microcentrifuge tube. If desired, keep the pellet and process it alongside the insoluble fraction; this end product is the *unclarified fraction*.
13. Spin at 100,000g for 20 minutes (fixed-angle TLA-55 rotor at 40,309 rpm, 4°C, in a Beckman Coulter Optimax tabletop ultracentrifuge).
14. Decant supernatant into a 1.5mL microcentrifuge tube: this is the *soluble fraction*.
15. Take 10ul aliquot of soluble fraction and mix with Laemmli buffer; use this to run a protein gel and assess protein integrity.
16. Mix soluble fraction with 1/10 vol 2% SDS, and equal volume Phenol, to denature proteins and begin RNA extraction.

Insoluble fraction extraction

17. Violently snap pellet to clear remaining liquid.
18. Add 500 μl soluble RNA buffer (SRB) and vortex violently. (The pellet may not resuspend; that's fine.)
19. Spin at 100,000g for 20 minutes.
20. Discard supernatant, clear residual liquid with a hard snap.
21. Add 500 μl insoluble RNA buffer (IRB). Vortex briefly.
22. Add equal volume Phenol pH 8, vortex until pellet dissolves, begin RNA extraction.

RNA extraction

Extract using standard phenol:chloroform method.

23. Having mixed equal volumes of aqueous solution with Phenol, ensure tubes are vortexed thoroughly.
24. Spin at 14,000g for 2mins at 4°C.
25. Transfer aqueous phase (roughly 200μl) to new 1.5ml tube (labeled tube N), avoiding cloudy interphase and lipids on top. Add 250μl SPB to previous tube (labeled tube A) and vortex tube A for 5mins.
26. Add 250μl chloroform to tube P to suck off phenol from water phase. Vortex for 3 mins, then spin 2mins at 14,000g and transfer aqueous phase from tube A to tube N. Discard tube A.
27. Repeat extraction on tube N: mix with equal volume Phenol:Chloroform pH 4.5, vortex 30s, 14,000g for 2mins, remove aqueous phase to new tube. Repeat until interphase is no longer cloudy.
28. To final aqueous sample add 1/10 vol ammonium Acetate, 1.5 vol 100% EtOH, and 1μl GlycoBlue (use a master mix!). Mix gently, precipitate overnight at -20°C.
29. Remove the sample from freezer. Cold spin (4°C) for 15 mins at 14,000g.
30. Thoroughly remove ethanol from pellet, and add 700μl 80% ethanol. Cold spin for 2 minutes at 14,000g. Repeat the ethanol wash and cold spin. This removes all traces of salt, SDS, etc.
31. Dry pellets thoroughly, i.e., pipette off ethanol, removing all liquid. If necessary, dry with the tubes open on a 37°C heat block (if the RNA sample is pure, this should not degrade the RNA). Resuspend pellet in 50 μl H_2O.
32. To check the quality of the RNA, pour a 1% agarose-TBE gel on RNA-free equipment, and run using NEB RNA loading dye. Heat loading dye and H_2O to 95°C for 5 minutes, and then cool, to reduce the possibility of contamination. Mix 1μl sample, 10μl 1X loading dye for each well. Perform a 2X serial dilution of the sample for more precise quantification.