Measurement of protein aggregation by fractionating yeast

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This protocol is for harvesting of yeast and separation of their proteins into aggregate/membrane and soluble fractions, for further analysis by SDS-PAGE, western blot, mass spectrometry, etc. Based on Mike Dion and Allan Drummond's 2011 protocols, with modifications by Edward Wallace[1] and Quincey Justman.

Prepare

- soluble protein buffer (SPB; 20mM HEPES-NaOH pH7.4, 120mM KCl, 2mM EDTA, 0.2mM DTT, 1:100 PMSF, 1:100 protease inhibitors cocktail IV). Make stock of salt and buffer, add DTT and inhibitors shortly before use, and chill.
- total protein buffer (TPB; 20mM HEPES-NaOH pH7.4, 150mM NaCl, 5mM EDTA, 3% SDS, 1:100 PMSF, 2mM DTT, 1:1000 protease inhibitors IV).
- insoluble protein buffer (IPB; 8M Urea, 20mM HEPES-NaOH pH7.4, 150mM NaCl, 2% SDS, 2mM EDTA, 2mM DTT, 1:100 PMSF, 1:1000 protease inhibitors IV.). Mix fresh daily the urea, DTT, and inhibitors, and keep at room temperature:

for 2mL	for 13mL	
950μL	6.2mL	H2O
75μL	390μL	5M NaCl
8μL	52μL	0.5M EDTA
200μL	1.3mL	20% SDS (w/v)
40μL	260μL	1M HEPES-NaOH pH7.4

Or make IPB stock from above ingredients, and use:

1.27 mL	8.2mL	IPB Stock
0.96g	6.24g	urea
20μL	130μL	100mM PMSF
4μL	26μL	1M DTT
2μL	13μL	protease inhibitors

Use 500μL IPB per sample, and note that Urea is slow to dissolve, and foaming will make ~25% of the solution unusable. IPB solidifies at 4°C due to urea and SDS; also, don't substitute KCl as it precipitates SDS even at RT.

- For heat treatment, pre-warm water bath to 46°C or pre-warm media as required.
- Appropriate numbers of safe-lok tubes loaded with 7mm steel balls, racked in liquid nitrogen (LN2).
- Pre-chill a centrifuge, also label and lay out all tubes and equipment in advance as the protocol moves very quickly once started.
- Protocol can be paused and samples stored at -80°C when cells have been flash-frozen (step 7), and after cells have been ground in mixer mill (step 10).

Sample Growth

1. Grow up a culture of Saccharomyces cerevisiae (BY4741) to 4×10^6 cells/mL ($OD_{600} \approx 0.45$) at 30°C, with 120rpm shaking, in 300ml SC-complete medium in a 1000ml erlenmeyer flask.

[1] Previous versions of this file were called solubleInsolubleProtein*.*

Sample Lysis

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 in a swinging bucket rotor at RT. The end of this spin marks the start of the timed treatment duration. Gently decant and discard supernatant. For heat shock treatment, hold tube containing pellet in waterbath at desired temperature for desired time; alternatively, transfer to pre-warmed media for desired time and end by spinning as described.
4. Resuspend pellet in 1ml ice-cold SPB, on ice, and transfer to 1.5ml tube.
5. Spin at 5,000g, 4°C, for 30 seconds.
6. Resuspend new pellet in 150μL lysis buffer.
7. Drip 100uL of resuspended pellet onto upper wall of tube containing steel ball, still racked in LN2. 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. Place the remaining 100uL of resuspended pellet in a tube for total protein extraction: process, or freeze, immediately.
8. 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.)
9. 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.
10. Remove sample tubes from LN2, tap on bench to release powder from lid, and pop the caps to relieve pressure.
11. Add 400 μL SPB to each tube, thaw on ice with occasional vortexing, and as soon as possible extract ball with a magnet. (We rinse balls in methanol and store in 50% ethanol.)

Soluble fraction extraction

12. Spin at 3000g for 30 seconds (clarification step) to remove whole cells and very large aggregates.
13. 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*.
14. 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).
15. Decant supernatant into a 1.5mL microcentrifuge tube: this is the *soluble fraction*.
16. Take 10ul aliquot of soluble fraction and mix with Laemmli buffer; use this to run a protein gel and assess protein integrity.

Insoluble fraction extraction

17. Violently snap pellet to clear remaining liquid.
18. Add 500 μL soluble protein buffer (SPB) 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 250 μL insoluble protein buffer (IPB); note this means insoluble sample is relatively 2X concentrated to others. Process samples in IPB at room temperature to maintain solubility of the Urea.
22. Dislodge the pellet with a pipet tip, Vortex until pellet dissolves, 10-15 minutes for clarified samples.

23. Spin at 20,000g, RT, for 5 minutes.
24. Decant supernatant into a 1.5mL microcentrifuge tube: this is the *insoluble fraction*.
25. Run a 4-15% SDS-page gel; load roughly 5-10μL of total and soluble fractions and 2-4 times the quantity of insoluble fractions. Make aliquots for further analysis.

Total protein extraction

26. Add 400 μL TPB to each total protein tube. Incubate at 95C with 500rpm mixing for 20 min.
27. Vortex vigorously for 15 min.
28. Spin at 6000g, RT, 1 min. Take supernatant; this is the *total protein fraction*.

Chloroform:Methanol Extraction

For Mass Spectrometry runs, or shipping proteins at room temperature, first perform a precipitation. Detergents, like SDS, and salts, like NaCl, can disrupt LC-MS/MS runs. Precipitation with chloroform and methanol results in dry protein material, free of salt and detergent. Adapted from Wessel, D. and Flugge, U.I. (1984) Anal. Biochem. 138 141–143.

29. To 100μL protein sample (100μg protein) in a 1.5mL tube:
30. Add 400μL methanol and vortex thoroughly.
31. Add 100μL chloroform and vortex.
32. Add 300μL H2O—mixture will become cloudy with precipitate—and vortex.
33. Centrifuge 1 minute at 14,000g. Result is three layers: a large aqueous layer on top, a circular flake of protein in the interphase, and a smaller chloroform layer at the bottom.
34. Remove top aqueous layer carefully, trying not to disturb the protein flake.
35. Add 400μL methanol and vortex.
36. Centrifuge 5 minutes at 20,000g, which will slam dandruffy precipitate against the tube wall.
37. Remove as much methanol as possible. Be careful, because the pellet is delicate. You should be able to remove all but a few μL of methanol with care, which will speed drying.
38. Dry under vacuum, and seal tube. This may be stored at RT short-term or -20°C long term, prior to resuspension.