Protein extraction of soluble and insoluble fractions from E. coli

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This protocol is for harvesting of E. coli and separation of their proteins into soluble and insoluble fractions, for further analysis by SDS-PAGE, western blot, mass spectrometry, etc. Based on Mike Dion and Allan Drummond's 2011 yeast protocols, with modifications by Edward Wallace and Matt Champion. This version forked after yeast version 2c.

Prepare

- MOPS EZ Rich Defined Medium (Teknova M2105)
- soluble protein buffer (SPB; 20mM HEPES-NaOH pH7.4, 120mM KCl, 2mM EDTA, 2mM DTT, 1:100 PMSF, 1:100 protease inhibitors cocktail IV.) Make stock of salt and buffer, and add DTT and inhibitors shortly before use, and chill.
- insoluble protein buffer (IPB; 8M Urea, 20mM HEPES-NaOH pH7.4, 150mM NaCl, 2% SDS, 10mM 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	
920μL	6.0mL	H2O
75μL	390μL	5M NaCl
40μL	260μL	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.
- total protein buffer (TPB; 20mM HEPES-NaOH pH7.4, 150mM NaCl, 2mM EDTA, 0.2mM DTT, 1:100 PMSF, 1:100 protease inhibitors cocktail IV.) Make stock of salt and buffer, and add DTT and inhibitors shortly before use, and chill.
- Vacuum filtration apparatus
- 50mL conical tube with holes pierced in bottom, in dewar of LN.
- Appropriate numbers of safe-lok tubes loaded with 7mm steel balls, racked in liquid nitrogen (LN2).
- Ice-water bath and sonicator with small tip.
- Label and lay out all tubes and equipment in advance as the protocol moves very quickly once started. Pause points are when cells have been flash-frozen, and after cells have been ground in mixer mill.

Sample Growth

1. Streak out a culture of *Escherichia coli* (MG1655) on an LB plate incubated at 37°C, for 12hours or overnight. Place the plate at 4°C for up to 24hrs if necessary, once colonies are grown up (~ .5mm wide).
2. Inoculate a single culture from the plate into 2mL MOPS medium, and grow to saturation at 30C overnight.

3. Make a 1:250 dilution and grow up to $OD_{600} \approx 0.3$ ($\approx 2 \times 10^8$cells/mL) at 30°C, with 225rpm shaking: put 1mL of the overnight culture in 250ml MOPS medium in a 1000ml erlenmeyer flask. Growth takes about 3h.

Sample Lysis for Soluble/Insoluble Fractionation

4. Fill filter with preheated media. Turn on vacuum. Pour entire liquid culture onto filter. Scrape with cell scraper (Costar 3008) until sufficient material

5. Plunge cell pellet, on scraper, into conical tube of LN use another chilled cell scraper to displace pellet from first scraper. Place tube at -80°C; when all LN2 has boiled out of tube (listen – if any popping or hissing, keep waiting), transfer cell pellet to reinforced eppendorf tube, with pre-chilled tweezers.

6. Snap tube closed carefully, away from other tubes. Keep in LN2.

7. 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.

8. Remove sample tubes from LN2, tap on bench to release powder from lid, and pop the caps to relieve pressure.

9. 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.)

10. Sonicate (Fisher FM120) for 20s in 5s pulses, 20kHz, 40% amplitude, with the tube suspended in ice-water bath. This step is to shear genomic DNA and reduce viscosity prior to sample clarification. (Alternatively, vortex 4 times on high in 10sec pulses, placing the sample on ice between pulses).

11. Take 20uL aliquot as total protein sample.

Soluble fraction extraction

12. Spin at 10,000g for 30 seconds (clarification step) to remove whole cells and very large aggregates.

13. Gently remove whitish lipid layer floating on top, and discard. 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 500 μL insoluble protein buffer (IPB). 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*. If desired, keep the pellet, this is the *leftover fraction*; boil the leftover thoroughly in Laemmli buffer before running a gel.

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 and continue to chloroform:methanol extraction.

Lysis and extraction for total protein

26. Transfer 50 mL of the cell culture cells to a 50mL conical tube.
27. Spin at 4,000g for 2mins in a swinging bucket rotor at 4°C. Gently discard the supernatant.
28. Resuspend pellet in 1ml ice-cold TPB, on ice, and transfer to 1.5ml tube.
29. Spin at 10,000g, 4°C, for 1min.
30. Resuspend new pellet in total of 1mL TPB.
31. Sonicate at at 20% intensity, 7 seconds on 7 seconds off, for 5mins, in ice bath.
32. Add 1/10 vol of 20% SDS. Vortex. Boil 5 mins. Place on ice.
33. Spin 30s, 10,000g, 4°C, to clear membranes and debris. Take aliquot for SDS-PAGE, and continue to chloroform:methanol extraction.

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 Flgge, U.I. (1984) Anal. Biochem. 138 141143.

34. To 100μL protein sample ($\sim$ 100μg protein) in a 1.5mL tube:
35. Add 400μL methanol and vortex thoroughly.
36. Add 100μL chloroform and vortex.
37. Add 300μL H2O—mixture will become cloudy with precipitate—and vortex.
38. 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.
39. Remove top aqueous layer carefully, trying not to disturb the protein flake.
40. Add 400μL methanol and vortex.
41. Centrifuge 5 minutes at 20,000g, which will slam dandruffy precipitate against the tube wall.
42. Remove as much methanol as possible. Be careful, because the pellet is delicate. You should be able to remove all but a few uL of methanol with care, which will speed drying.
43. Dry under vacuum, and seal tube. This may be stored at RT short-term or -20 long term, prior to resuspension.