Thiol Disulfide exchange protocol

Extracting yeast mitochondria from the culture (Meisinger, Pfanner, & Truscott, 2006)

1. Check that samples reach an OD600nm of ~0.8

2. Centrifuge 50mL of yeast culture at 3000g for 5 mins

3. Discard the supernatant

4. Resuspend and wash pellet with ~300mL of pure water

5. Centrifuge at 3000g for 5 mins and discard the supernatant

6. Resuspend pellet with 5mL pre-warmed DTT buffer 100 mM Tris-H2SO4, pH 9.4, 10 mM dithiothreitol (DTT), prewarmed to 30°C. Add DTT prior to use. A 1 M Tris-H2SO4 stock can be stored at room temperature.

7. Shake yeast and DTT buffer mixture slowly at 30°C for 20 mins

8. Centrifuge at 3000g for 5 mins and discard supernatant

9. Resuspend pellet in 25mL zymolyase buffer

10. Centrifuge at 3000g for 5 mins and discard supernatant

11. Resuspend pellet in 25mL zymolyase buffer

12. Shake solution slowly at 30oC for 30-45 mins

13. Centrifuge at 3000g for 5 mins and discard supernatant

14. Resuspend pellet in 25mL zymolyase buffer

15. Centrifuge at 3000g for 5 mins and discard supernatant

16. Resuspend pellet in 30mL ice-cold homogenisation buffer (0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2% (w/v) bovine serum albumin (BSA; essentially fatty acid-free, Sigma-Aldrich, Taufkirchen, Germany). Add PMSF from a freshly prepared 100 mM stock in ethanol just prior to use.)

17. Using a Dounce homogeniser, homogenise the spheroplasts with ~15 strokes at 4oC

18. Use 60mL of homogenisation buffer to wash out and collect as much of the sample as possible into a falcon tube

19. Centrifuge homogenate at 1500g for 5 mins at 4oC to pellet cell debris and nuclei. Collect the supernatant

20. Centrifuge the supernatant at 4000g for 5 mins at 4oC. Collect the supernatant and discard the pellet. Collect 50uL aliquots from each sample for 1D gel and quantitation.

21. Centrifuge the supernatant at 12 000g for 15 mins at 4oC. Keep the pellet

22. Resuspend the mitochondrial pellet in SEM (50 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2)

23. To store the sample, use liquid nitrogen to flash freeze and store at -80oC

Protein TDE (Paulech et al., 2013; Rookyard et al., 2020)

1. Resolubilise the pellet with 0.1 M NaH2PO4 1% SDS 20 mM NEM 2 mM DTPA and vortex for 10 minutes to promote protein denaturation and alkylation

2. Quench NEM with 25 mM DTT for 15 minutes at room temperature

3. Raise pH >7 and reduce protein for 1 hour at 25oC

4. Perform chloroform methanol protein precipitation as described below (Rookyard et al., 2020; Whittend, 2009)

(1) Take 200µL aliquots of supernatant

(2) Add 4 volumes of ice cold methanol (800µL)

(3) Vortex well

(4) Add 1 volumes of ice cold chloroform (200µL)

(5) Vortex well

(6) Add 3 volumes of ice cold water (600µL)

(7) Vortex well

(8) Bench top spin at 14 000 g for 5 mins at 4 °C

(9) Remove as much of the upper phase as possible (discard) without disrupting the protein interface

(10)Add 3 volumes of ice cold methanol (600µL)

(11)Vortex well

(12)Bench top spin at 14 000 g for 5 mins at 4 °C

(13)Remove supernatant and discard

(14)Wash pellet using ice-cold methanol (600µL)

(15)Vortex well

(16)Bench top spin at 14 000 g for 5 mins at 4 °C

(17)Remove supernatant and discard

5. Use Thiopropyl-sepharose 6B beads and rinse with Milli-Q water (2mL/10mg beads).

6. Equilibrate with beads with 2 x 1 mL 100 mM NaH2PO4 pH 7.5 1 % w/v SDS 1 mM DTPA

7. Resuspend proteins in loading buffer to 1 mg / mL (100 mM NaH2PO4, pH 7.5 1% w/v SDS 1 mM DTPA)

8. Bind proteins 400 ug / 40 mg beads for 2 hours with gentle tumbling (ensure some protein is left for 'unenriched')

9. Wash once with 1 mL loading buffer for 10 minutes

10. Wash twice with 100 mM NaH_2PO_4, pH 7.5 0.5 % w/v SDS for 10 minutes

11. Wash 6 times with 100 mM NaH_2PO_4, pH 7.5 for 5 minutes.

12. Elute proteins with 5 x 200 uL of 10 mM DTT in 10 mM NaH_2PO_4, pH 7.5 for 15 minutes each elution.

13. Reduce unenriched and unbound fractions whilst waiting for the bound fraction

14. Alkylate all fractions with 50 mM IAA in the dark

15. Concentration TDE-bound proteins by molecular weight using a 3000 MWCO cut-off filter for 30 mins at 12 000g.

SDS-PAGE (Brunelle & Green, 2014a, 2014b)

1. Load 15 uL in each well (assuming that the concentrations of proteins obtained in the fraction are of the same concentration. If not, load the appropriate amount to ensure equal concentrations between bound, unbound and unenriched samples)

2. Run gel at 150V for ~40 mins or until the dye front has run off the gel

3. Stain gel with Coomassie blue stain on a shaker for 10mins

4. Destain gel by rinsing gel with water in the tray

5. Add destaining solution and heat for 10-20 secs in microwave then leave on the shaker for 10 mins

6. Image destained gel using imaging software

Once proteins at bands of interest have been identified, cut out bands of interest and prepare for mass spectrometry (MS) analysis. Use MS to identify proteins of interest.

References

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