Preparation of Mitochondria from *Candida albicans*

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[Abstract] Based on the methods of (Daum *et al.*, 1982) and (Hewitt *et al.*, 2012), we have established the use of *Candida albicans* as a new model system to study mitochondrial biogenesis. This dimorphic yeast provides an excellent system to investigate the coordination of mitochondrial biogenesis with other cellular networks including cellular metabolism and the cell cycle. Unlike the model lab yeast *Saccharomyces cerevisiae*, which has been widely used in the mitochondrial biogenesis field, *C. albicans* is not subject to the Crabtree effect, hence grows aerobically in glucose when oxygen is present. Therefore the control of mitochondrial biogenesis in *C. albicans* is more typical of eukaryotic cells. *C. albicans* has a fully sequenced genome and there are many published tools for genetic manipulation facilitating Systems Biology approaches. The isolation of mitochondria as described in this protocol produces a more simplified system that can be interrogated using the standard tools of molecular biology. In addition the import of radiolabelled proteins as described in the protocol: *Candida albicans* Mitochondrial Protein Import Assay (Hewitt *et al.*, 2013) is a sensitive technique that can be used to determine details of kinetics and interactions of imported proteins.

Materials and Reagents

1. *Candida albicans* culture
2. Tris-SO_4
3. DTT
4. Sorbitol
5. Lyticase (Sigma-Aldrich, catalog number: L2524)
6. K^+MES [Potassium 2-(N-morpholino)ethanesulfonate]
7. K^+HEPES [Potassium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate]
8. PMSF
9. Tris-DTT buffer (see Recipes)
10. Sorbitol buffer (see Recipes)
11. KH_2PO_4 & K_2HPO_4 (see Recipes)

12. Breaking Buffer (BB) 6.0 (see Recipes)

Equipment

1. Incubator shaker with temperature control
2. Centrifuge
3. Dounce (tight dounce 40 ml) (Wheaton, catalog number: 06-435C)

Procedure

A. Culture preparation

Inoculate with sufficient starter culture to produce a culture of *Candida albicans* cells with an OD_{600} = 1.0-2.0 (an OD_{600} of up to 7 has worked in our hands) at the desired start time the next morning. Shake at 200 rpm at 30 °C overnight.

B. Method

1. Collect cells by spinning at 4,000 *x g*/10 min/RT (room temperature).
2. Pour off medium.
3. Resuspend cells in dH_2O in weighed centrifuge tubes.
4. Spin at 2,500 *x g*/5 min/RT. Reweigh to get mass of cells.
5. Resuspend cells in Tris-DTT buffer (see recipe) ~5 ml per g of cells.
6. Incubate for 15 min at 30 °C with gentle shaking (~100 rpm).
7. Spin at 2,500 *x g*/5 min /RT.
8. Resuspend pellet in ~5 ml/g of cells pre-warmed 1.2 M sorbitol buffer.
9. Spin at 2,500 *x g*/5 min /RT.
10. Weigh out 0.2 mg/g of cells of lyticase. Dissolve in 2 ml pre-warmed (30 °C) 1.2 M sorbitol buffer per gram of cells. Resuspend pellet in this solution.
11. Incubate cells in lyticase solution for ~60 min/30 °C with gentle shaking (~100 rpm) or until spheroplasts form (check spheroplast formation by osmotic shock: Add 30 µl cells to 2 ml 1.2 M sorbitol and water. After vortexing water sample should go clear).
12. Spin down for 2,500 *x g*/5 min /RT. Discard supernatant
13. Resuspend in cold 1.2 M sorbitol buffer ~5 ml/g of cells. Spin 2,500 *x g*/5 min/4 °C.
14. Resuspend in minimal amount of cold Breaking Buffer (BB) 6.0 then make up in BB 6.0 containing 1 mM PMSF final (~4 ml/g cells).
15. Homogenize 15 times using a tight dounce. Dounce should be ~¾ full or a bit less. Up stroke must be fast and steady. Bubbles can break mitochondria so try not to let it pop out.

16. Spin homogenate 5 min/4 °C/3,000 *x g*. Save supernatant in a new tube and keep on ice (Supernatant should be cloudy).
17. (Optional step for higher yield) Resuspend pellet again in BB 6.0 with PMSF and tight dounce and spin as above. Combine supernatants. Discard pellets.
18. Spin combined supernatants 5 min/4 °C/3,000 *x g*. Save supernatant (repeat this spin step to clear more of the contaminating membranes if still getting a large pellet).
19. Spin supernatant for 10 min/4 °C/12,000 *x g*.
20. Pour off the supernatant and resuspend pellet in a small amount of BB 7.4 (with bovine serum albumin if mitochondria are to be used for *in vitro* import assays).
21. Spin down 10 min/4 °C/12,000 *x g* then remove any white membranes that surround reddish-brown mitochondrial pellet before resuspending in a minimal amount of BB 7.4 with BSA.
22. Repeat spin step above if still contaminating membranes present.
23. Estimate the mitochondrial concentration as follows: Add 10 µl crude mitochondria to 990 µl 0.6% SDS. As a blank add 10 µl BB 7.4 with BSA to 990 µl 0.6% SDS. Measure A_{280} using Quartz cuvette. An absorbance value of 0.21 corresponds to 10 mg/ml protein in the undiluted mixture.
24. Aliquot at appropriate volumes and snap freeze in dry ice/liquid nitrogen.

Recipes

1. Tris-DTT
   0.1 M Tris-SO_4 (pH 9.4)
   10 mM DTT (make fresh from frozen 1 M DTT aliquots just before use)
2. 1.2 M sorbitol buffer
   1.2 M sorbitol
   20 mM KPi (pH7.4)
3. BB (6.0) breaking buffer
   0.6 M sorbitol
   20 mM K^+MES (pH 6.0) (add PMSF just prior to use to 1 mM final concentration)
4. Phenylmethanesulfonylfluoride (PMSF)
   34 mg/ml (0.2 M) in ethanol
5. BB (7.4) breaking buffer
   0.6 M sorbitol
   20 mM K^+HEPES (pH 7.4) (adjust pH with KOH)
6. 2.4 M sorbitol stock (aqueous solution deionised)
7. 1 M K^+MES stock (pH 6.0)

Filter sterilize and store in foil at room temperature

8. 1 M KPi (pH 7.4) make from stocks of 1 M KH_2PO_4 and stock of 1 M K_2HPO_4 and mix them until pH = 7.4.
   Do not titrate with HCl or NaOH.

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References

1. Daum, G., Gasser, S. M. and Schatz, G. (1982). Import of proteins into mitochondria. Energy-dependent, two-step processing of the intermembrane space enzyme cytochrome b2 by isolated yeast mitochondria. *J Biol Chem* 257(21): 13075-13080.
2. Hewitt, V. L., Heinz, E., Shingu-Vazquez, M., Qu, Y., Jelicic, B., Lo, T. L., Beilharz, T. H., Dumsday, G., Gabriel, K., Traven, A. and Lithgow, T. (2012). A model system for mitochondrial biogenesis reveals evolutionary rewiring of protein import and membrane assembly pathways. *Proc Natl Acad Sci U S A* 109(49): E3358-3366.
3. Hewitt, V., Lithgow, T. and Traven, A. (2013). *Candida albicans* Mitochondrial Protein Import Assay. *Bio-protocol* 3(4): e331.